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(54) Title: PURIFIED pH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC ACIDS ENCODING SAME															
(57) Abstract															
<p>The present invention relates to isolated nucleic acid fragments containing a sequence encoding a <i>Rhizoctonia solani</i> laccase having optimum activity at a neutral or basic pH, and the laccase proteins encoded thereby.</p>															

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PURIFIED PH NEUTRAL RHIZOCTONIA LACCASES AND NUCLEIC
ACIDS ENCODING SAME

5

Related Applications

This application is a continuation-in-part of co-pending U.S. Serial Nos. 08/122,230, 08/122,827, and 08/162,827, the contents of which are incorporated by reference in their entirety.

10 15 Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, which functions at a neutral pH.

20 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and

basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively 5 from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial applications. Among these are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice 10 manufacture, phenol resin production, and waste water treatment.

Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ 15 depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for several of these have been cloned. For example, Choi et al. (*Mol. Plant-Microbe Interactions* **5**: 119-128, 1992) describe the molecular characterization and cloning of the 20 gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima et al. (*J. Biol. Chem.* **265**: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch 25 (*Experientia* **41**: 801, 1985; *PNAS USA* **83**: 8854-8858, 1986) have reported the cloning and partial sequencing of the *Neurospora crassa* laccase gene. Saloheimo et al. (*J. Gen. Microbiol.* **137**: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the 30 fungus *Phlebia radiata*. However, virtually all of the known fungal laccases function best at acidic pHs (e.g., between pH 3.0 and 6.0), and are typically inactive at

neutral or basic pHs. Since a number of the aforestated potential industrial methods are preferentially conducted at neutral or basic pH, most fungal laccases perform poorly in such methods. Thus, the available fungal laccases are 5 inadequate for application in a number of important commercial methods.

An exception to this rule is the extracellular laccase produced by certain species of *Rhizoctonia*. Bollag et al. have reported a laccase with a pH optimum of about 7.0 10 produced by *Rhizoctonia praticola*. A laccase of this type would be far more useful in industrial methods requiring neutral pH than previously known laccases. However, the *R. praticola* enzyme was neither purified nor further characterized, nor, to date, has any other laccase having 15 this trait been purified or characterized. Moreover, although other laccase genes have been isolated, as described above, these have been genes encoding enzymes which function best at acidic pH. Recombinant production and commercially adequate yields of a pH neutral or basic 20 laccase have thus been unattainable due to the fact that neither the enzyme per se nor the laccase gene encoding such an enzyme has previously been isolated and/or purified and sequenced. The present invention now provides a solution to each of these problems.

25

Summary of the Invention

The present invention relates to an isolated nucleic acid fragment comprising a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at a pH 30 between 6.0 to 8.5. By "functioning optimally" is meant that the enzyme exhibits significant (i.e., at least about 30% of maximum, preferably at least about 50%, and most

preferably from 50% to maximum) activity within the pH range of between about 6.0-8.5, as determined by activity in one or more standard laccase assays for substrates such as the syringaldazine, ABTS, 2,6-dimethoxyphenol, or 4
5 antiaminopyrine + N-ethyl-N-sulfonybutyl-m-toluidine. A preferred substrate for the laccases of the present invention is syringaldazine. In a preferred embodiment, the laccase is a *Rhizoctonia solani* laccase. The invention also relates to a substantially pure laccase encoded by the novel
10 nucleic acid sequence. By "substantially pure" is meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells
15 comprising the claimed nucleic acid fragment, which vectors and host cells are useful in recombinant production of the laccase. The nucleic acid fragment is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of
20 choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the nucleic acid fragment of the invention, or progeny thereof, under
25 conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin
30 manipulation, juice manufacture, phenol polymerization and phenol resin production. In a preferred embodiment, the

enzyme of the invention is used in a process requiring a neutral or somewhat basic pH for greatest efficiency.

Brief Description of the Figures

5 Figure 1 illustrates the nucleotide and amino acid sequence of RSlac1. Lower case letters in the nucleotide sequence indicate the position of introns.

10 Figure 2 illustrates the nucleotide and amino acid sequence of RSlac2. Lower case letters in the nucleotide sequence indicate the position of introns.

15 Figure 3 illustrates a restriction map of the plasmid pMWR-1.

20 Figure 4 illustrates the nucleotide and amino acid sequence of the translated region of RSlac3.

25 Figure 5 illustrates the syringaldazine oxidase activity of RSlac1 (90mM buffer, 20 µM syringaldazine, 20°C).

30 Figure 6 illustrates the syringaldazine oxidase activity of RSlac2 (93mM buffer, 20 µM syringaldazine, 20°C).

Detailed Description of the Invention

Certain species of the genus *Rhizoctonia* have been reported as producing laccase; therefore, an initial search focused on identifying the presence of these enzymes in 25 various *Rhizoctonia solani* isolates. Samples are cultured and the supernatants periodically analyzed for the presence of laccase by the ABTS method, described below. Laccase is observed in all the *Rhizoctonia* cultures. Harvested laccases are electrophoretically separated and stained with 30 ABTS. One isolate, RS22, produces a laccase with a basic pI, and is selected for further study.

The remaining studies focus on purification and characterization of the enzyme from RS22. Briefly, the fermentation broth is filtered and concentrated by UF with a membrane cut off of about 10,000. A first ion exchange chromatography step is conducted at pH 4.5 in acetate buffer, with step elution using NaCl. The eluate is then ultrafiltered and rechromatographed, and eluted with a NaCl gradient. Active fractions are pooled for further study.

5 The intact protein thus isolated and purified
10 (hereinafter referred to as RSlac3) is first subjected to partial sequencing, and the N-terminal sequence obtained is as follows:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

15 The protein is further subjected to digestion with a lysine- or glutamic-acid specific protease, and additional peptides obtained from the protein have the following sequences, which can be aligned with sequences in *Coriolus hirsutus*:

Peptide 1:

20 SQYVDGLRGPLVIYDPDDDH (SEQ. ID. NO: 6)

Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRYBVBBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

25 Peptide 4:

SLGPTPNVNPXIRDVVVRGGTTVV (SEQ. ID. NO. 9)

The following peptides are also found, but do not correspond to *Coriolus* sequences

Peptide 5:

30 IRYVGGAVAL(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)

Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

In the above sequences, B designates a residue which is either aspartic acid or asparagine, and X designates 5 unidentified residues.

In order to initiate screening for a *Rhizoctonia* laccase gene, an *R. solani* genomic library is prepared. Total DNA is partially digested with restriction enzyme Sau3A, and electrophoresed in an agarose gel to isolate DNA 10 fragments between 8 and 21 kb in size. The fractionated fragments are ligated to λ phage EMBL3 arms with BamHI ends, and the resulting phage packaged *in vitro*. These phage are used as a library to create a library of 170,000 plaques in *E. coli* and amplified 100-fold for future use.

15 In order to develop probes for isolation of the *R. solani* laccase gene, the protein sequences of five known laccases are analyzed to determine consensus sequences, and two degenerate oligonucleotides constructed based on observed consensus sequences (Choi et al. *supra*; Germann and 20 Lerch, *supra*; Saloheimo et al, *supra*, Kojima et al, *supra*). These oligos are mixed with *R. solani* genomic DNA and a DNA fragment of 220 nucleotide fragment is amplified using a taq polymerase chain reaction(PCR). The 220-nucleotide fragment is then cloned into plasmid vector.

25 The PCR fragment is used as a probe to screen 25,000 plaques from the amplified genomic library. Positive clones from this screen fall into two classes that are subsequently shown, by DNA sequence analysis, to code for two different laccase genes, RSlac1 and RSlac2. The nucleotide sequence 30 for each of these genes (SEQ ID. NOS.: 1 and 3), and the predicted amino acid sequence for each protein (SEQ. ID. NOS.: 2 and 4), are presented in, respectively, Figures 1

and 2. The homology between the two sequences is approximately 63%. Compared to known laccase sequences from *Coriolus hirsutus*, *Phlebia radiata*, *Aspergillus nidulans*, *Cryphonectria parasitica*, and *Neurospora crassa*, the RS 5 laccases show between about 30-40% homology. Each of the two coding sequences is cloned into an expression vector operably linked to *Aspergillus oryzae* taka-amylase transcription and translation signals (See Figure 3). Each of the two laccase expression vectors is transformed into an 10 *Aspergillus oryzae* and *Aspergillus niger* host cell, and the host cells screened for the presence of laccase.

For isolation of the RSlac3 gene, polyA RNA is purified from *R. solani* mycelia grown in the presence of anisidine. The RNA is used as a template for cDNA synthesis. The cDNA 15 is fractionated and fragments between 1.7-3.5 kb collected, and a cDNA library created by cloning the fractionated DNA into a yeast vector. 3000 transformants from this library are screened on ABTS. After 24 hours, a single colony appears positive. The plasmid from the colony is isolated 20 and the insert sequenced. Portions of the predicted amino acid sequence correspond with the sequences of the fragments obtained from RS 22, described *supra*. The complete nucleotide and amino acid sequences are depicted in Figure 4, and in SEQ. ID. NOS.: 13 and 14, respectively. RSlac3 25 shows 48% homology with RSlac1 and 50% homology with RSlac2. RSlac3 also shows 48% homology with the *Coriolus hirsutus* laccase gene.

According to the invention, a *Rhizoctonia* gene encoding a pH neutral or basic laccase can be obtained by 30 methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression

vector. A useful expression vector contains an element that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and

5 preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To

10 permit the secretion of the expressed protein, nucleotides encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be treated according to the invention is operably linked to the

15 control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad.

20 Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

25

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it

30 is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is

independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host
5 cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may
10 be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention,
15 especially in a bacterial host, are the promoter of the lac operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the
20 promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase,
25 *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* or *A. awamii* glucoamylase (*gluA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred
30 are the TAKA-amylase and *gluA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention.

5 Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19,
10 pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. li-*
15 *cheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD* and *SC*, a marker giving rise to hygromycin resistance. Preferred for use in an
20 *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

25

It is generally preferred that the expression is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable,
30 this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the

DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from

- 5 *Rhizomucor miehei*, the gene for the α -factor from *Saccharomyces cerevisiae* or the calf prochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TAKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B.*
- 10 *stearothermophilus* α -amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TAKA amylase signal, the *Rhizomucor miehei* aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

- 15 The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance,
- 20 Sambrook et al. *Molecular Cloning*, 1989).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the

- 25 recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more
- 30 likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed

according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

5

The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lenthus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus laetus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may be selected from a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of

transforming *Fusarium* species is described by Malardier et al., 1989.

The present invention thus provides a method of producing a recombinant laccase of the invention, which 5 method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in 10 question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae (e.g. in catalogues of the American Type Culture Collection).

15 The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed 20 by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, 25 juice or detergent applications.

In a particularly preferred embodiment, the expression of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid 30 containing the *Aspergillus oryzae* TAKA α -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker. Alternatively, the *amdS* may be on a separate plasmid and

used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Yelton et al. (PNAS USA 81: 1470-1474, 1984).

5 Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figures 1 and 2. It will be apparent that the invention also encompasses those nucleotide sequences that encode the
10 same amino acid sequences as depicted in Figures 1, 2 and 3, but which differ from those specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. In addition, the invention also encompasses other nucleotide fragments, and the proteins encoded thereby, which encode
15 laccase proteins having substantially the same pH optimum as those of *Rhizoctonia solani*, and which show a significant level of homology with the *Rhizoctonia solani* amino acid sequence. For example, the present data show that more than one species of *Rhizoctonia* produces a laccase with the
20 desired pH profile; it is therefore expected that other *Rhizoctonia* species also produce similar laccases and therefore, using the technology described herein, can be used as a source for genes within the scope of the claimed invention. As also shown in the present examples, not only
25 is there more than one nucleotide and amino acid sequence that encodes a laccase with the required characteristics, there is also considerable variation tolerated within the sequence while still producing a functional enzyme. Therefore, the invention also encompasses any variant
30 nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80% homology with one or the other of the amino acid sequences depicted in Figures 1,

2 and 3, and retains both the laccase and pH optimum activity of the sequences described herein. In particular, variants which retain a high level(i.e., $\geq 80\%$) of homology at highly conserved regions of the *Rhizoctonia* laccase are 5 contemplated. Such regions are identified as residues 458-469 in RSLAC1, and 478-489 in RSLAC2; and residues 131-144 in RSLAC1 and 132-145 in RSLAC2.

Useful variants within the categories defined above include, for example, ones in which conservative amino acid 10 substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, 15 and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the 20 regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be determined by conducting a standard ABTS oxidation method in 0.1M sodium phosphate at pH 7.0.

The protein can be used in number of different 25 industrial processes; although the enzyme is also functional to some extent at lower pH, the *R. solani* laccase is most beneficially used in processes that are usually conducted at a neutral or alkaline pH, since other laccases are not active in this pH range. These processes include 30 polymerization of lignin, both Kraft and lignosulfates, in solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a

particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., Holzforschung 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992.

5 The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the
10 production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., Svensk papperstidning 5: 162-166, 1976.
15 Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

Oxidation of dyes and other chromophoric compounds
20 leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for dye transfer inhibition
25 and dye oxidation can be found in WO 92/01406, WO 92/18683, EP 0495836 and Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversiteit Gent. 56: 1565-1567, 1991.

The present laccase can also be used for the
30 polymerization of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a

precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, *Fruit processing* 7/93, 248-252, 1993; Maier et al., *Dt. Lebensmittelrindschau* 86(5): 137-142, 1990; Dietrich et al., *Fluss. Obst* 57(2): 67-73, 1990. The invention is further illustrated by the following non-limiting examples.

EXAMPLES

1. Purification and characterization of *R. solani* laccase

10 Individual isolates of *R. solani* cultured on potato dextrose agar (Difco) are examined for laccase enzyme formation by transferring a small piece of agar containing vigorous growth to 100 ml CFM (24.0 g potato dextrose broth, 3.0 g yeast extract, 1.0 ml Microelement solution
15 [0.80 g KH₂PO₄, 0.64 g CuSO₄·5H₂O, 0.11 g FeSO₄·7H₂O, 0.80 g MnCl₂·4H₂O, 0.15 g ZnSO₄·7H₂O, distilled water to 1000 ml], distilled water to 1000 ml) in a 500 ml shake flask. Incubation is at room temperature, at 200 rpm on an orbital shaker.

20 Samples are harvested at 50, 74, 122 and 170 hours, centrifuged and the clear supernatant analyzed for laccase with its ABTS (ABTS= 2,2'-azinobis (3 ethylbenzothiazoline-6-sulfonic acid). The analysis is carried out by adding 200 µl of 2mM ABTS in 0.1 M phosphate buffer, pH 7, and
25 observing the change in absorbance at 418 nm after 30 minutes incubation at room temperature (approximately 23-25° C). This method is modified from a peroxidase analysis method described by Pütter and Becker (*Peroxidases*, in: Bergmeyer, H.U.(ed.), *Methods of Enzymatic Analysis*, 3rd
30 ed., Vol.III, pp.286-293, 1983)

Each of the laccases harvested at 172 hours is electrophoretically separated and stained with ABTS as

chromogen. Several distinct patterns emerge; the strain RS 22 is shown to produce a laccase having a basic pI, and is chosen for further characterization.

Laccase activity is also determinable by the 5 syringaldazine method. Laccase catalyzes the oxidation of syringaldazine to tetramethoxy azo bis-methylene quinone under aerobic conditions, with a change of color from yellow to violet. 3000 μ l of 25 mM acetate buffer (containing 10mg/l cuprisulfate, 5 H₂O) at pH 5.5, 30°C, is mixed in a 1 10 cm cuvette with 225 μ l 0.28 mM syringaldazine (5mg solubilized in 25 ml ethanol and adjusted to 50 ml with demineralized water). The mixture is then mixed with 100 μ l of a laccase dilution (diluted in acetate buffer so that the increase in absorbance (Δ OD) is within the range of 0.1-0.6). 15 The reaction mixture is placed in a 30°C thermostated spectrophotometer and the reaction is followed at 530 nm for 10 to 70 seconds from the addition of laccase. The activity of the enzyme is calculated as Δ OD/minute \times 0.677 \times dilution factor, and is expressed as LACU.

20 For purification of the *Rhizoctonia* laccase, 2.1 liter of culture medium with a LACU activity of 0.19 LACU/ml is filtered through a 10 μ m filter and concentrated to 230 ml by ultrafiltration using a Filtron Minisette OMEGA membrane with a cutoff value of 10 kDa. The pH of the sample is 5.3 25 and the activity of the concentrated sample is determined to be 3.34 LACU/ml.

After pH adjustment to 4.5 and filtration due to slight precipitation, the sample is applied to a 40 ml S Sepharose Fast Flow column equilibrated with 20mM acetate buffer at pH 30 4.5 (buffer A). The column is washed in buffer A and eluted with buffer A containing 1 M NaCl. Active fractions are collected and pooled. This active pool is concentrated and

buffer exchanged to buffer A using an Amicon ultrafiltration unit equipped with a Diaflo YM10 membrane. This sample is rechromatographed on a 5 ml S Sepharose High Performance column using the method described above except that elution 5 is carried out with a linear gradient over 30 column volumes from buffer A to buffer A containing 0.5 M NaCl. The fractions from this purification exhibiting highest activity are pooled. Approximately 45 mg laccase are obtained, when protein concentration is estimated by one absorption unit at 10 A280 nm equal to 1mg/ml. The protein is >90% pure as judged by SDS-PAGE. The molecular weight estimated by SDS-PAGE is approximately 67 kDa. The specific activity of the purified protein is 1 LACU/mg. The pH profile of the purified protein, using syringaldazine as substrate is show in Table 15 1, below.

Table 1.

pH	5	6	7	8
20 % activity	0.5	31	100	59

For sequencing of the protein, peptides are generated using wither a lysine-specific protease from *Achromobacter* (*Achromobacter* protease I) or a glutamic acid specific 25 protease from *Bacillus licheniformes*. The peptides are purified by reverse phase HPLC employing linear gradients of 80% 2-propanol containing 0.08% aqueous TFA (solvent B) in 0.1% aqueous TFA (solvent A).

N-terminal amino acid sequence analysis of the intact 30 protein and of purified peptides are carried out in an Applied Biosystems 473A protein sequencer according to the manufacturer's instructions. Initial partial sequencing of

the isolated protein yields the following N-terminal sequence:

AVRNYKFDIKNVNVAPDGFQRPIVSV (SEQ. ID. NO.: 5)

The protein is then digested with either a lysine- or 5 glutamic-acid specific protease, and following additional peptides identified. Peptides 1-4 can be aligned with sequences in the laccase of *Coriolus hirsutus*:

Peptide 1:

SQYVDGLRGPLVIVDPDDDH (SEQ. ID. NO: 6)

10 Peptide 2:

GLALVFAEAPSQIRQGVQSVQPDDA (SEQ. ID. NO.: 7)

Peptide 3:

SRYBVBBASTVVMLEBWYHTPAXVLE (SEQ. ID. NO. 8)

Peptide 4:

15 SLGPTPNVVNPXIRDVVRRGGTTVV (SEQ. ID. NO. 9)

Peptide 5:

IRYVGGA^VX(N?)RSVI (SEQ. ID. NO.: 10)

Peptide 6:

ILANPA (SEQ. ID. NO.: 11)

20 Peptide 7:

YEAPSLPT (SEQ. ID. NO.: 12)

An X in the above sequences designates an unidentified residue, and B represents a residue which is either aspartic acid or asparagine.

25

2. Isolation of *R. solani* laccase gene

A study of the known amino acid sequences of fungal laccases obtained from non-*Rhizoctonia* species (Choi et al., supra; German et al., supra; Saloheimo et al. supra; and 30 Kojima et al, supra) is conducted to determine the presence of consensus sequences among them. Two regions of high identity, IHWHGFFQ and TFWYHSH, are found near the amino

terminal third of the protein. Based on these consensus sequences and the corresponding DNA sequences, three degenerate oligonucleotides, O-lac2 [TGG/AAAGACCATA/GGTGTCG/AGTA/G], its complement O-lac2r, and 5 O-lac3 [ATCCAT/CTGGCAT/CGGG/CA/TTCTTCCAG/A], are synthesized using an Applied Biosystems 394 DNA/RNA synthesizer.

The synthesized oligos are used in a polymerase chain reaction (PCR) to screen *Rhizoctonia solani* genomic DNA for a laccase gene or fragment thereof. For amplifications of 10 genomic DNA, 0.5 µg of genomic DNA is incubated with 1µM of each primer, 200µM of dNTPs, and 1 U taq polymerase (Boehringer Mannheim) in [10 mM Tris-Cl, 1.5 mM MgCl₂, 50 mM KCl, 1 mg/ml gelatine; pH 8.3]. The reactions are incubated for 1x5 minutes at 95°C, 30x[1 minute at 95°C, 1 minute at 15 50-60°C, 1 minute at 72°C], and 1x5 minutes at 72°C. The PCR reactions amplify a DNA fragment of 220 nucleotides. The PCR product is cloned, according to manufacturer's directions, into the TA cloning vector (InVitrogen Corp.). Characterization of the PCR product by DNA sequencing of 20 individual clones distinguishes two separate laccase genes designated RSlac1 and RSlac2.

To prepare a *R. solani* genomic library, *R. solani* DNA is partially digested with restriction enzyme Sau3A, and electrophoresed through a 0.8% Sea Plaque Agarose (FMC Bioproducts) in a Tris/Acetate/EDTA buffer to isolate those 25 DNA fragments between 8.0 an 21 kb in size. The gel fractionated fragments are further purified with Beta-Agarase (New England Biolabs) according to manufacturer's instruction, and then ligated to lambda phage EMBL3 arms 30 with BamHI ends. The resulting phages are packaged in vitro using Gigapack II packaging extract (Stratagene). 25 ml of TB media+0.2% maltose and 10 MgSO₄ is inoculated into a 50 µl

aliquot of an overnight culture of *E. coli* K802 (supE, hsdR, gal, metB) and incubated at 37°C with shaking until the A600=0.5. 25 µl of a 1:10 and 1:50 dilution of the packaged phage are mixed with 250 µl of the K802 cells, and incubated 5 for 20 minutes at 37°C. To each dilution, 5 µl of melted top agar at 48°C are added. The mix is then plated onto prewarmed LB plates and incubated at 37°C for at least 12 hours. From these phage, a library of 170,000 plaques in *E. coli* K802 is created and amplified 100-fold for future 10 use.

To screen for the laccase gene, 25,000 plaques from the amplified genomic library are plated onto NZY/agarose plates for plaque lifts using conventional methods. Filters are screened using the 220 nucleotide PCR fragment randomly 15 labelled to 5×10^8 cpm/µg as a probe. Filters are hybridized in 50% formamide, 6xSSC for 16 hours at 42°C and washed with 0.5xSSC, 0.1% SDS at 65°C. Positive clones are picked and rescreened using conventional methods. The nine positive 20 clones identified fell into two classes that by DNA sequence analysis are shown to code for two different laccase genes, RSlac1 and RSlac2. The complete nucleotide sequence of each of these genes is determined using fluorescent nucleotides and an Applied Biosystems automatic DNA sequencer (Model 363A, version 1.2.0). The nucleotide and predicted amino 25 acid sequences are depicted in Figures 1 and 2.

For isolation of RSlac3, poly A RNA purified from *R. solani* mycelia grown in the presence of 1 mM anisidine is used as a template for cDNA synthesis using standard protocols. The cDNA is fractionated by electrophoresis 30 through a 0.8% agarose gel and DNA fragments between 1.7 and 3.5 kb in size are collected. A library is then created by cloning the size-fractionated cDNA into the yeast expression

vector pYES2. 3000 yeast transformants from this library are plated initially on YNB (1.7 g yeast nitrogen base without amino acids, 5 g $(\text{NH}_4)_2\text{SO}_4$ per liter) with 2% glucose. After 4 days growth at 30°C, the resulting 5 colonies are replica plated to YNB with 0.1% glucose, 2% galactose and 2mM ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid; Sigma # A-1888). After 24 hours of growth at 30°C a single colony has a light green halo which gradually turns a dark purple. The plasmid from 10 this colony is isolated and the insert sequenced. The sequence of the translated portion of the RSlac3 gene and protein is shown in SEQ.ID NOS. 13 and 14, and in Figure 4.

3. Expression of laccase gene

The plasmid PMWR-1 is a pUC derived vector containing 15 the TAKA amylase transcription regulation signals and the TAKA amylase signal sequence. This plasmid is engineered with a unique SfiI site at the signal sequence cleavage site, and a 3' adjacent NsiI site such that these two restriction enzymes can be used to introduce, in frame, a 20 foreign protein. Using a PCR reaction (conducted as described above, but with 100 ng of the appropriate linearized plasmid DNA as a template) and mutagenized primers, an SfiI site is introduced at amino acid 12 and amino acid 14 of RSlac1 and RSlac2, respectively, such that 25 the protein coding sequences are in frame with the TAKA signal sequence. In addition, a PCR amplification is also used to introduce a PstI site (CTGCAG) at the 3' end of RSlac1 and an NsiI site (ATGCAT) at the 3' end of RSlac2.

30 To prepare for transformation, cells of *Aspergillus oryzae* are cultivated in YPG (1g/l yeast extract, 0.25 g K_2PO_4 , 0.125 g/ MgSO_4 , 3.75 g glucose) at 34°C with 100-120rpm

for 16-20 hours, then collected by filtration with miracloth. Cells are washed with Mg solution (0.6M MgSO₄·7H₂O), then 2-6 g of cells are taken up in 10 ml MgP(1.2M MgSO₄·7H₂O, 10mM NaH₂PO₄·2H₂O; pH 5.8). To this is
5 added 1 ml of Novozyme® 234 (120 mg/ml MgP), and the sample kept on ice for 5 minutes. One ml of BSA (12 mg/ml) is added, and the sample shaken gently at 34-37°C. Protoplasts are collected by filtration through miracloth, and overlain with 5 ml of ST (0.6 M Sorbitol, 100mM Tris; pH 7). The
10 sample is spun at 2500 rpm for 15 minutes, and a band of protoplasts collected. Two volumes of STC (1.2M Sorbitol, 10 mM tris, 10 mM CaCl₂·2H₂O; pH 7.5) are added and the sample is spun at 2500 rpm for 5 minutes. The precipitate is washed twice with 5 ml of STC, and the protoplasts suspended in
15 0.5-1ml of STC.

For the transformation process, the protoplast concentration is adjusted to 1-5x10⁷/ml. To 100 µl of protoplast solution is added a maximum of 10 µl of DNA solution (5-10 µg of supercoiled DNA) and 0.2 ml of PEG
20 (60% PEG4000, 10mM Tris, 10mM CaCl₂·H₂O; pH 7.5), and the combination is mixed well. The sample is kept at room temperature for 25 minutes; then to it is added first 0.2 ml PEG, with mixing, the 0.85 ml PEG with mixing. The mixture is kept at room temperature for 20 minutes, then spun at
25 4000 rpm for 15 minutes. The precipitate is washed with 2 ml of STC by spinning at 2500 rpm for 10 minutes. The protoplasts are resuspended in 0.2-0.5 ml of STC, and then spread on COVE plates. COVE medium (pH 7) contains 342.3 g/l sucrose, 25 g/l agar and a salt solution comprising 26 g/l
30 KCl, 26 g/l MgSO₄·H₂O, 76 g/l KH₂PO₄, and 50 ml/l of trace metals; the trace metals are 40 mg/l NaB₄O₇·10H₂O, 400 mg/l

CuSO₄·5H₂O, 1200mg/l FeSO₄·7H₂O, 700mg/l MnSO₄·H₂O, 800mg/l Na₂MoO₄·2H₂O, 10 g/l ZnSO₄·7H₂O). After autoclaving, 10 ml/l of 1M filtrated acetamide and 5-10 ml of 3M CsCl are added to the solution. Transformants are selected by growth cells 5 on COVE medium which contains acetamide as the carbon source.

The confirmation of laccase production in the samples is determined by the ABTS oxidation method as described above on Cove medium with 2 mM ABTS, at pH 5 and 7.3. Both 10 RSlac1 and RSlac2 express laccase activity at pH 5 and pH 7, in contrast with a control laccase which shows substantially no activity at pH 7.3.

The products of the expression of each of RSlac1 and RSlac2 are tested for oxidase activity at various pHs using 15 syringaldazine as the substrate. The assay is conducted substantially as described above for the assay of the native protein, over pH range of 4-9. As shown in Figures 5 and 6, both laccases are active at pHs over pH 5, and RSlac1 has particularly good activity at pHs over 6. The pattern of 20 activity is generally comparable to that observed for the RSlac3 laccase isolated from RS 22 (see Table 1 above), with RSlac1 exhibiting the broadest range of activity.

Deposit of Biological Materials

The following biological materials have been deposited 25 under the terms of the Budapest Treaty in the International Mycological Institute, Genetic Resource Reference Collection, located at Bakeham Lane, Egham, Surrey TW20 9TY and given the following accession number.

30 Deposit Accession Number
Rhizoctonia solani RS22 IMI CC 358730

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria,

5 Illinois, 61604 and given the following accession numbers.

<u>Deposit</u>	<u>Accession Number</u>
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E. coli containing RSlac1 fused to an α -amylase signal sequence (EMCC 00844)	NRRL B-21141
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10

E. coli containing RSlac2 with an SfiI site insert (EMCC 00845)	NRRL B-21142
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15

E. coli containing RSlac3 (EMCC 0088)	NRRL B-21156
--	--------------

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: PURIFIED PH NEUTRAL LACCASES AND NUCLEIC ACIDS ENCODING SAME

(iii) NUMBER OF SEQUENCES: 14

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: to be assigned
- (B) FILING DATE: 13-SEP-1994

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/172,331
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(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/122,230
- (B) FILING DATE: 17-SEP-1993

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/122,827
- (B) FILING DATE: 17-SEP-1993

(vii) PRIOR APPLICATION DATA:

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- (B) FILING DATE: 03-DEC-1993

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2838 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 302..351

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 463..512

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 576..633

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(B) LOCATION: 760..818

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(B) LOCATION: 822..877

(ix) FEATURE:
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(B) LOCATION: 1001..1054

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(ix) FEATURE:
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(B) LOCATION: 1697..1754

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(A) NAME/KEY: intron
(B) LOCATION: 1992..2051

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2157..2206

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2348..2404

(ix) FEATURE:

(A) NAME/KEY: intron
 (B) LOCATION: 2438..2498

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: join(170..301, 352..462, 513..575, 634..759, 819
 ..821, 878..1000, 1055..1315, 1373..1696, 1755
 ..1826, 1881..1991, 2052..2156, 2207..2347, 2405
 ..2437, 2499..2621)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CGC ACC ACT TTC CTT GTC TCG GTT TCG CTC TTT GTT TCC GCT GTT CTT Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala Val Leu 5 10 15	223
GCG CGC ACC GTC GAG TAC GGC TTG AAG ATT AGT GAT GGG GAG ATA GCT Ala Arg Thr Val Glu Tyr Gly Leu Lys Ile Ser Asp Gly Glu Ile Ala 20 25 30	271
CCT GAC GGT GTT AAG CGT AAT GCG ACT TTG GTACGCCACTC CTGTAAATCC Pro Asp Gly Val Lys Arg Asn Ala Thr Leu 35 40	321
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GGT AGC CAT CAT CTG CAT TCG CGC AGC GTC GTT AAG CGC CAG AAT GAG	1660

Gly Ser His His Leu His Ser Arg Ser Val Val Lys Arg Gln Asn Glu 355 360 365	
ACC ACC ACT GTT GTA ATG GAC GAG AGC AAG CTC GTT GTAAAGTACCA Thr Thr Thr Val Val Met Asp Glu Ser Lys Leu Val 370 375 380	1706
TATTTAAAAG TTGGTTGGGT TTCGAATACT TATTTCAACT TTTCTTAG CCA CTG GAA Pro Leu Glu	1763
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CTC ACT TTT GGT TTG GTATGTAGCC AAATCGCCCA TATACAGGAT ACTGAATATT Leu Thr Phe Gly Leu 405	1866
GTTCGTGCGT GTAG AAC TTT GCT ACC GGG CAC TGG ATG ATC AAC GGT ATC Asn Phe Ala Thr Gly His Trp Met Ile Asn Gly Ile 410 415	1916
CCA TAC GAG TCT CCC AAA ATC CCC ACA TTG CTC AAG ATC CTC ACT GAT Pro Tyr Glu Ser Pro Lys Ile Pro Thr Leu Leu Lys Ile Leu Thr Asp 420 425 430	1964
GAG GAC GGG GTT ACC GAG TCT GAC TTC GTATGTTCCC TTTTCGGTAT Glu Asp Gly Val Thr Glu Ser Asp Phe 435 440	2011
CTTCGTATGC GTGCACTGAC TCGTGCTGGT GGGAAATTAG ACC AAG GAG GAG CAC Thr Lys Glu Glu His 445	2066
ACA GTC ATA CTC CCG AAG AAC AAA TGC ATC GAA TTC AAC ATC AAG GGG Thr Val Ile Leu Pro Lys Asn Lys Cys Ile Glu Phe Asn Ile Lys Gly 450 455 460	2114
AAC TCG GGT ATT CCC ATT ACG CAC CCC GTA CAT CTT CAC GGT Asn Ser Gly Ile Pro Ile Thr His Pro Val His Leu His Gly 465 470 475	2156
GTAAAGTCAT ATCGGATGGT TTACGATACT AAGGCTCATC AACTTTTAG CAC ACT His Thr	2212
TGG GAT GTC GTA CAA TTT GGC AAC AAC CCA CCC AAT TAT GTC AAT CCT Trp Asp Val Val Gln Phe Gly Asn Asn Pro Pro Asn Tyr Val Asn Pro 480 485 490 495	2260
CCC CGT AGG GAC GTG GTT GGC TCT ACA GAT GCG GGT GTG AGG ATT CAG Pro Arg Arg Asp Val Val Gly Ser Thr Asp Ala Gly Val Arg Ile Gln 500 505 510	2308
TTC AAG ACC GAC AAT CCA GGA CCG TGG TTC CTG CAC TGC GTGCGTCGGT Phe Lys Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys 515 520	2357
CCCCATCGTC CGTTATGGTT TTTCTAATAC GTCCCCATTCT ATTTTAG CAT ATT GAC His Ile Asp 525	2413
TGG CAT CTT GAG GAG GGT TTC GCA GTGAGTACTG AGACCTAAGT GCTACTCGGC Trp His Leu Glu Glu Gly Phe Ala 530 535	2467

TCATTAATGAGA TTACCGCATG TATGCCCTCA G ATG GTG TTT GCT GAA GCG CCC Met Val Phe Ala Glu Ala Pro 540	2519
GAA GCC GTC AAG GGA GGT CCA AAG AGC GTG GCC GTG GAC TCT CAG TGG Glu Ala Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp 545 550 555	2567
GAA GGG CTG TGT GGC AAG TAC GAC AAC TGG CTA AAA TCA AAT CCG GGC Glu Gly Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly 560 565 570	2615
CAG CTG TAGGCGTATC GCAGCCACAT TGGTGATGAT TGAAAGTTGC ATCTTGTCC Gln Leu 575	2671
TATAACCGGC TCTTATATAC GGGTGTCTCC CAGTAAAGTC GTAGCCCAAT TTCAGCCGAG ACAGATATTT AGTGGACTCT TACTCTTGTG TCCCATTGAC GCACATCGTT GCATCAAACC TGCTTTTAT CGTCCCTCTT TGTAATTGTG GTTGCTGTAA TGTATCG	2731 2791 2838

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 576 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Arg Thr Thr Phe Leu Val Ser Val Ser Leu Phe Val Ser Ala 1 5 10 15
Val Leu Ala Arg Thr Val Glu Tyr Gly Leu Lys Ile Ser Asp Gly Glu 20 25 30
Ile Ala Pro Asp Gly Val Lys Arg Asn Ala Thr Leu Val Asn Gly Gly 35 40 45
Tyr Pro Gly Pro Leu Ile Phe Ala Asn Lys Gly Asp Thr Leu Lys Val 50 55 60
Lys Val Gln Asn Lys Leu Thr Asn Pro Glu Met Tyr Arg Thr Thr Ser 65 70 75 80
Ile His Trp His Gly Leu Leu Gln His Arg Asn Ala Asp Asp Asp Gly 85 90 95
Pro Ser Phe Val Thr Gln Cys Pro Ile Val Pro Arg Glu Ser Tyr Thr 100 105 110
Tyr Thr Ile Pro Leu Asp Asp Gln Thr Gly Thr Tyr Trp Tyr His Ser 115 120 125
His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile 130 135 140
Tyr Asp Pro Lys Asp Pro His Arg Arg Leu Tyr Asp Val Asp Asp Glu 145 150 155 160
Lys Thr Val Leu Ile Ile Gly Asp Trp Tyr His Glu Ser Ser Lys Ala 165 170 175

Ile Leu Ala Ser Gly Asn Ile Thr Arg Gln Arg Pro Val Ser Ala Thr
 180 185 190
 Ile Asn Gly Lys Gly Arg Phe Asp Pro Asp Asn Thr Pro Ala Asn Pro
 195 200 205
 Asp Thr Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg Tyr Arg Leu
 210 215 220
 Arg Val Ile Asn Ser Ser Glu Ile Ala Ser Phe Arg Phe Ser Val Glu
 225 230 235 240
 Gly His Lys Val Thr Val Ile Ala Ala Asp Gly Val Ser Thr Lys Pro
 245 250 255
 Tyr Gln Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg Ile Asp Cys
 260 265 270
 Val Val Glu Ala Asn Gln Glu Pro Asp Thr Tyr Trp Ile Asn Ala Pro
 275 280 285
 Leu Thr Asn Val Pro Asn Lys Thr Ala Gln Ala Leu Leu Val Tyr Glu
 290 295 300
 Glu Asp Arg Arg Pro Tyr His Pro Pro Lys Gly Pro Tyr Arg Lys Trp
 305 310 315 320
 Ser Val Ser Glu Ala Ile Ile Lys Tyr Trp Asn His Lys His Lys His
 325 330 335
 Gly Arg Gly Leu Leu Ser Gly His Gly Leu Lys Ala Arg Met Ile
 340 345 350
 Glu Gly Ser His His Leu His Ser Arg Ser Val Val Lys Arg Gln Asn
 355 360 365
 Glu Thr Thr Thr Val Val Met Asp Glu Ser Lys Leu Val Pro Leu Glu
 370 375 380
 Tyr Pro Gly Ala Ala Cys Gly Ser Lys Pro Ala Asp Leu Val Leu Asp
 385 390 395 400
 Leu Thr Phe Gly Leu Asn Phe Ala Thr Gly His Trp Met Ile Asn Gly
 405 410 415
 Ile Pro Tyr Glu Ser Pro Lys Ile Pro Thr Leu Leu Lys Ile Leu Thr
 420 425 430
 Asp Glu Asp Gly Val Thr Glu Ser Asp Phe Thr Lys Glu Glu His Thr
 435 440 445
 Val Ile Leu Pro Lys Asn Lys Cys Ile Glu Phe Asn Ile Lys Gly Asn
 450 455 460
 Ser Gly Ile Pro Ile Thr His Pro Val His Leu His Gly His Thr Trp
 465 470 475 480
 Asp Val Val Gln Phe Gly Asn Asn Pro Pro Asn Tyr Val Asn Pro Pro
 485 490 495
 Arg Arg Asp Val Val Gly Ser Thr Asp Ala Gly Val Arg Ile Gln Phe
 500 505 510
 Lys Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys His Ile Asp Trp
 515 520 525
 His Leu Glu Glu Gly Phe Ala Met Val Phe Ala Glu Ala Pro Glu Ala

530	535	540
Val Lys Gly Gly Pro Lys Ser Val Ala Val Asp Ser Gln Trp Glu Gly		
545	550	555
Leu Cys Gly Lys Tyr Asp Asn Trp Leu Lys Ser Asn Pro Gly Gln Leu		
565	570	575

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3117 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Rhizoctonia laccase

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: join(393..524, 577..687, 737..799, 860..985, 1043 ..1045, 1097..1219, 1269..1538, 1601..1996, 2047 ..2118, 2174..2284, 2338..2439, 2495..2635, 2693 ..2725, 2786..2899)

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 525..576

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 688..736

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 800..859

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 986..1042

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1220..1268

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1539..1600

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1823..1936

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1973..2046

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 2119..2173

(ix) FEATURE:

- (A) NAME/KEY: intron

(B) LOCATION: 2285..2337

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2440..2494

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 2636..2692

(ix) FEATURE:

(A) NAME/KEY: intron
(B) LOCATION: 1046..1096

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAGTGATCCG CCAGAGTTCA GGCGGATAAG TTCCCTAAATA GTCATTGCC TATTCGTGTA	60
CCTCAGCATA CTGACGACAT ACCGCCAGAT CGCCCTCGGT TCGGGCGTGG CATACTTCG	120
CAAGGGCACC TCACCGAGCA AACTCTAAA AGCTTCGGCA TGGAATTGCAT TTTGTATTGT	180
AAACAAGTTA CGAGAAAAAC AATAGATCAG TTTTGCCGA ATCGGATGGC TTGAAACGGA	240
AGTACCGATG CCCGATCCGA GTCGAATGAA TTAACCCATC TGAAACGGGA CCCTGAGTCG	300
AGGCACCCGC CGGCCTTGGC CGTATAAGTC ACTTGTGCC AACTAGCACT TTTTCATTCC	360
CCCTTTCTT CTTCTCGTC TTCTTCTCT CT ATG GCT CGG TCG ACT ACT TCA	413
Met Ala Arg Ser Thr Thr Ser	
1 5	
CTC TTT GCA CTG TCT CTC GTT GCT TCA GCG TTT GCT CGA GTC GTT GAC	461
Leu Phe Ala Leu Ser Leu Val Ala Ser Ala Phe Ala Arg Val Val Asp	
10 15 20	
TAT GGG TTT GAT GTG GCT AAT GGG GCA GTT GCT CCG GAT GGT GTA ACA	509
Tyr Gly Phe Asp Val Ala Asn Gly Ala Val Ala Pro Asp Gly Val Thr	
25 30 35	
AGG AAC GCG GTT CTC GTGAGTTAGC TGTAAGATGG TGTATATGCT GGTTGCCTAA	564
Arg Asn Ala Val Leu	
40	
CGGGAATGTC AG GTC AAT GGT CGC TTC CCT GGT CCA TTG ATC ACC GCC	612
Val Asn Gly Arg Phe Pro Gly Pro Leu Ile Thr Ala	
45 50 55	
AAC AAG GGG GAT ACA CTT AAA ATC ACC GTG CGG AAT AAA CTC TCC GAT	660
Asn Lys Gly Asp Thr Leu Lys Ile Thr Val Arg Asn Lys Leu Ser Asp	
60 65 70	
CCA ACT ATG CGA AGG AGC ACG ACC ATC GTTAGTACTT CCCCTCATCT	707
Pro Thr Met Arg Arg Ser Thr Thr Ile	
75 80	
GTCTTGAAAC TTTCTCATCT TTTTGAAAG CAC TGG CAC GGT CTG CTC CAA CAC	760
His Trp His Gly Leu Leu Gln His	
85	
AGG ACG GCA GAA GAA GAT GGC CCG GCC TTT GTA ACC CAG GTATGCCTTA	809
Arg Thr Ala Glu Glu Asp Gly Pro Ala Phe Val Thr Gln	
90 95 100	
TCCTATCGCT GCTCTGTCCC CGCGTCCTTC CCTGACTCGG GCGATTCTAG TGC CCG	865
Cys Pro	

ATT CCT CCG CAA GAA TCG TAC ACC TAT ACG ATG CCG CTC GGC GAA CAG Ile Pro Pro Gln Glu Ser Tyr Thr Tyr Thr Met Pro Leu Gly Glu Gln 105 110 115 120	913
ACC GGC ACG TAT TGG TAC CAC AGC CAC TTG AGC TCC CAG TAT GTG GAC Thr Gly Thr Tyr Trp Tyr His Ser His Leu Ser Ser Gln Tyr Val Asp 125 130 135	961
GGG TTG CGT GGG CCC ATC GTT ATT GTAAGTCTTC ATTAAACCTT ATTCTTGTT Gly Leu Arg Gly Pro Ile Val Ile 140	1015
ATGGCTGATT GTGACGTCGT GGTTAGT ATG TTCTGGCTT CCACAAGAAG Met 145	1065
TCAGCAGCCC TTGAAGCTAA CTTTATTCCA G GAC CCC CAC GAC CCG TAC AGA Asp Pro His Asp Pro Tyr Arg 150	1117
AAC TAC TAT GAT GTC GAC GAC GAG CGT ACG GTC TTT ACT TTA GCA GAC Asn Tyr Tyr Asp Val Asp Asp Glu Arg Thr Val Phe Thr Leu Ala Asp 155 160 165	1165
TGG TAC CAC ACG CCG TCG GAG GCT ATC ATT GCC ACC CAC GAT GTC TTG Trp Tyr His Thr Pro Ser Glu Ala Ile Ile Ala Thr His Asp Val Leu 170 175 180	1213
AAA ACG GTACGCGTTA ATCCTTCTAG CTTTCTTCC TTGGGTCACT TTCTATCAG Lys Thr 185	1268
ATC CCC GAC TCG GGT ACG ATC AAC GGC AAA GGC AAA TAC GAT CCT GCT Ile Pro Asp Ser Gly Thr Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala 190 195 200	1316
TCG GCT AAC ACC AAC ACG ACA CTC GAG AAC CTC TAC ACT CTC AAA Ser Ala Asn Thr Asn Asn Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys 205 210 215	1364
GTC AAA CGC GGC AAG CGG TAT CGC CTG AGG ATT ATC AAC GCC TCG GCC Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala 220 225 230	1412
ATC GCT TCG TTC CGG TTC GGC GTG CAG GGC CAC AAG TGC ACG ATC ATC Ile Ala Ser Phe Arg Phe Gly Val Gln Gly His Lys Cys Thr Ile Ile 235 240 245 250	1460
GAG GCT GAT GGC GTC CTC ACC AAA CCG ATC GAG GTC GAT GCG TTT GAT Glu Ala Asp Gly Val Leu Thr Lys Pro Ile Glu Val Asp Ala Phe Asp 255 260 265	1508
ATT CTA GCA GGC CAG AGG TAT AGC TGC ATC GTAAGTCTAC CTATGCCTTG Ile Leu Ala Gly Gln Arg Tyr Ser Cys Ile 270 275	1558
TTGTGGAGAT AAGAACCTGA CTGAATGTAT GCGCTCCAAT AG TTG AAG GCC GAC Leu Lys Ala Asp 280	1612
CAA GAT CCT GAT TCC TAC TGG ATA AAT GCG CCA ATC ACA AAC GTT CTC Gln Asp Pro Asp Ser Tyr Trp Ile Asn Ala Pro Ile Thr Asn Val Leu 285 290 295	1660
AAC ACC AAC GTC CAG GCA TTG CTA GTG TAT GAA GAT GAC AAG CGT CCT	1708

Asn Thr Asn Val Gln Ala Leu Leu Val Tyr Glu Asp Asp Lys Arg Pro			
300	305	310	
ACT CAC TAC CCC TGG AAG CCG TTT TTG ACA TGG AAG ATA TCA AAT GAA		1756	
Thr His Tyr Pro Trp Lys Pro Phe Leu Thr Trp Lys Ile Ser Asn Glu			
315	320	325	
ATC ATT CAG TAC TGG CAG CAC AAG CAC GGG TCG CAC GGT CAC AAG GGA		1804	
Ile Ile Gln Tyr Trp Gln His Lys His Gly Ser His Gly His Lys Gly			
330	335	340	
AAG GGG CAT CAT CAT AAA GTC CGG GCC ATT GGA GGT GTA TCC GGG TTG		1852	
Lys Gly His His His Lys Val Arg Ala Ile Gly Gly Val Ser Gly Leu			
345	350	355	360
AGC TCC AGG GTT AAG AGC CGG GCG AGT GAC CTA TCG AAG AAG GCT GTC		1900	
Ser Ser Arg Val Lys Ser Arg Ala Ser Asp Leu Ser Lys Lys Ala Val			
365	370	375	
GAG TTG GCT GCT GCA CTC GTT CGC GGT GAG GCC GAG TTG GAC AAG AGG		1948	
Glu Leu Ala Ala Leu Val Ala Gly Glu Ala Glu Leu Asp Lys Arg			
380	385	390	
CAG AAT GAG GAT AAT TCG ACT ATT GTA TTG GAT GAG ACC AAG CTT ATT		1996	
Gln Asn Glu Asp Asn Ser Thr Ile Val Leu Asp Glu Thr Lys Leu Ile			
395	400	405	
GTAAGTCCT TAATTTTTT CGGTGTCACG GAAGCTAACCC CGCGTAATAG CCG TTG		2052	
Pro Leu			
410			
GTT CAA CCT GGT GCA CCG GGC GGC TCC AGA CCA GCT GAC GTC GTG GTC		2100	
Val Gln Pro Gly Ala Pro Gly Ser Arg Pro Ala Asp Val Val Val			
415	420	425	
CCT CTG GAC TTT GGC CTC GTATGTGGCT TCTTGTTATT CGTCCGGAAT		2148	
Pro Leu Asp Phe Gly Leu			
430			
GCAAACGTAT TTGGGTGGGC TATAG AAC TTT GCC AAC GGA CTG TGG ACG ATA		2200	
Asn Phe Ala Asn Gly Leu Trp Thr Ile			
435	440		
AAC AAT GTC TCC TAC TCC CCT CCG GAT GTC CCT ACT CTC CTC AAG ATC		2248	
Asn Asn Val Ser Tyr Ser Pro Pro Asp Val Pro Thr Leu Leu Lys Ile			
445	450	455	
TTG ACC GAC AAA GAC AAA GTC GAC GCT TCT GAC TTC GTAGGTTCCCT		2294	
Leu Thr Asp Lys Asp Lys Val Asp Ala Ser Asp Phe			
460	465		
CTTCTTCTTT TCAAACTAGC TACTGACATT AAGTGAACGT CAG ACG GCC GAT GAA		2349	
Thr Ala Asp Glu			
470			
CAC ACG TAT ATT CTT CCA AAG AAC CAA GTT GTC GAG TTG CAC ATC AAG		2397	
His Thr Tyr Ile Leu Pro Lys Asn Gln Val Val Glu Leu His Ile Lys			
475	480	485	
GGA CAG GCT TTG GGA ATC GTA CAC CCC CTT CAT CTG CAT GGC		2439	
Gly Gln Ala Leu Gly Ile Val His Pro Leu His Leu His Gly			
490	495	500	
GTACGTCTTT CTCACACTGT TCCAGCTCCT ATTCTCTAAC ACACTCCTGC GATAG CAT		2497	
His			

GCG TTC GAC GTC GTC CAA TTC GGC GAC AAC GCT CCA AAC TAC GTG AAC Ala Phe Asp Val Val Gln Phe Gly Asp Asn Ala Pro Asn Tyr Val Asn 505 510 515 520	2545
CCT CCG CGT AGG GAT GTA GTA GGC GTA ACT GAT GCT GGA GTC CGT ATC Pro Pro Arg Arg Asp Val Val Gly Val Thr Asp Ala Gly Val Arg Ile 525 530 535	2593
CAG TTC AGA ACC GAT AAC CCG GGC CCT TGG TTC CTC CAT TGC Gln Phe Arg Thr Asp Asn Pro Gly Pro Trp Phe Leu His Cys 540 545 550	2635
GTATGCTCTT CATCTCCCAC CGCTTGTTCT TTACTTATGG TTTACCTTGC GATTTAG	2692
CAC ATT GAT TGG CAC TTG GAA GAA GGA TTT GCT GTAAGTTATT ATTCCTATT His Ile Asp Trp His Leu Glu Glu Gly Phe Ala 555 560	2745
CGAACGCATCG GGGAGATGCT AACCAAGGGT GTGTTTAAG ATG GTA TTC GCC GAA Met Val Phe Ala Glu 565	2800
GCG CCT GAA GAT ATC AAG AAA GGC TCT CAG AGT GTC AAG CCT GAC GGA Ala Pro Glu Asp Ile Lys Lys Gly Ser Gln Ser Val Lys Pro Asp Gly 570 575 580	2848
CAA TGG AAG AAA CTA TGC GAG AAG TAT GAG AAG TTG CCT GAA GCA CTG Gln Trp Lys Lys Leu Cys Glu Lys Tyr Glu Lys Leu Pro Glu Ala Leu 585 590 595	2896
CAG TGAAGTTGCA GTTGTTCACCC ATTGGAAAC TGGCTCACTA TTCCCTTTGCG Gln	2949
ATAATTCCGA CTTTTATTTT GGGACATTAT TGGACTATGG ACTTGTTTGT CACACCCTCG CTCACTGTGT CCCTCGTTGA GTACCTATAC TCTATTGTA TAGTGGGAAT ATGGAATATC	3009
GGATGTAATA AATGCTCGTG CGTTTGGTGC TCGAAATGGG GTAGGACT	3069
	3117

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 599 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Ala Arg Ser Thr Thr Ser Leu Phe Ala Leu Ser Leu Val Ala Ser
 1           5           10          15

Ala Phe Ala Arg Val Val Asp Tyr Gly Phe Asp Val Ala Asn Gly Ala
 20          25           30

Val Ala Pro Asp Gly Val Thr Arg Asn Ala Val Leu Val Asn Gly Arg
 35           40           45

Phe Pro Gly Pro Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Lys Ile
 50           55           60

Thr Val Arg Asn Lys Leu Ser Asp Pro Thr Met Arg Arg Ser Thr Thr
 65           70           75           80

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Ile His Trp His Gly Leu Leu Gln His Arg Thr Ala Glu Glu Asp Gly
 85 90 95
 Pro Ala Phe Val Thr Gln Cys Pro Ile Pro Pro Gln Glu Ser Tyr Thr
 100 105 110
 Tyr Thr Met Pro Leu Gly Glu Gln Thr Gly Thr Tyr Trp Tyr His Ser
 115 120 125
 His Leu Ser Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Ile Val Ile
 130 135 140
 Met Asp Pro His Asp Pro Tyr Arg Asn Tyr Tyr Asp Val Asp Asp Glu
 145 150 155 160
 Arg Thr Val Phe Thr Leu Ala Asp Trp Tyr His Thr Pro Ser Glu Ala
 165 170 175
 Ile Ile Ala Thr His Asp Val Leu Lys Thr Ile Pro Asp Ser Gly Thr
 180 185 190
 Ile Asn Gly Lys Gly Lys Tyr Asp Pro Ala Ser Ala Asn Thr Asn Asn
 195 200 205
 Thr Thr Leu Glu Asn Leu Tyr Thr Leu Lys Val Lys Arg Gly Lys Arg
 210 215 220
 Tyr Arg Leu Arg Ile Ile Asn Ala Ser Ala Ile Ala Ser Phe Arg Phe
 225 230 235 240
 Gly Val Gln Gly His Lys Cys Thr Ile Ile Glu Ala Asp Gly Val Leu
 245 250 255
 Thr Lys Pro Ile Glu Val Asp Ala Phe Asp Ile Leu Ala Gly Gln Arg
 260 265 270
 Tyr Ser Cys Ile Leu Lys Ala Asp Gln Asp Pro Asp Ser Tyr Trp Ile
 275 280 285
 Asn Ala Pro Ile Thr Asn Val Leu Asn Thr Asn Val Gln Ala Leu Leu
 290 295 300
 Val Tyr Glu Asp Asp Lys Arg Pro Thr His Tyr Pro Trp Lys Pro Phe
 305 310 315 320
 Leu Thr Trp Lys Ile Ser Asn Glu Ile Ile Gln Tyr Trp Gln His Lys
 325 330 335
 His Gly Ser His Gly His Lys Gly Lys Gly His His His Lys Val Arg
 340 345 350
 Ala Ile Gly Gly Val Ser Gly Leu Ser Ser Arg Val Lys Ser Arg Ala
 355 360 365
 Ser Asp Leu Ser Lys Lys Ala Val Glu Leu Ala Ala Ala Leu Val Ala
 370 375 380
 Gly Glu Ala Glu Leu Asp Lys Arg Gln Asn Glu Asp Asn Ser Thr Ile
 385 390 395 400
 Val Leu Asp Glu Thr Lys Leu Ile Pro Leu Val Gln Pro Gly Ala Pro
 405 410 415
 Gly Gly Ser Arg Pro Ala Asp Val Val Val Pro Leu Asp Phe Gly Leu
 420 425 430
 Asn Phe Ala Asn Gly Leu Trp Thr Ile Asn Asn Val Ser Tyr Ser Pro

435	440	445
Pro Asp Val Pro Thr Leu Leu Lys Ile Leu Thr Asp Lys Asp Lys Val		
450	455	460
Asp Ala Ser Asp Phe Thr Ala Asp Glu His Thr Tyr Ile Leu Pro Lys		
465	470	475
Asn Gln Val Val Glu Leu His Ile Lys Gly Gln Ala Leu Gly Ile Val		
485	490	495
His Pro Leu His Leu His Gly His Ala Phe Asp Val Val Gln Phe Gly		
500	505	510
Asp Asn Ala Pro Asn Tyr Val Asn Pro Pro Arg Arg Asp Val Val Gly		
515	520	525
Val Thr Asp Ala Gly Val Arg Ile Gln Phe Arg Thr Asp Asn Pro Gly		
530	535	540
Pro Trp Phe Leu His Cys His Ile Asp Trp His Leu Glu Glu Gly Phe		
545	550	555
Ala Met Val Phe Ala Glu Ala Pro Glu Asp Ile Lys Lys Gly Ser Gln		
565	570	575
Ser Val Lys Pro Asp Gly Gln Trp Lys Lys Leu Cys Glu Lys Tyr Glu		
580	585	590
Lys Leu Pro Glu Ala Leu Gln		
595		

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro			
1	5	10	15
Asp Gly Phe Gln Arg Pro Ile Val Ser Val			
20	25		

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro			
1	5	10	15
Asp Asp Asp His			
20			

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ser	Arg	Tyr	Asx	Val	Asx	Asx	Ala	Ser	Thr	Val	Val	Val	Met	Leu	Glu	Asx
1				5				10					15			
Trp	Tyr	Arg	Thr	Pro	Ala	Xaa	Val	Leu	Glu							
		20					25									

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ser	Leu	Gly	Pro	Thr	Pro	Asn	Tyr	Val	Asn	Pro	Xaa	Ile	Arg	Asp	Val
1				5				10				15			
Val	Arg	Val	Gly	Gly	Thr	Thr	Val	Val							
	20				25										

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Gly	Leu	Ala	Leu	Val	Phe	Ala	Glu	Ala	Pro	Ser	Gln	Ile	Arg	Gln	Gly
1				5				10				15			
Val	Gln	Ser	Val	Gln	Pro	Asp	Asp	Ala							
	20				25										

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ile Arg Tyr Val Gly Gly Pro Ala Val Xaa Arg Ser Val Ile
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Ile Leu Ala Asn Pro Ala
1           5

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(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 8 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Tyr Glu Ala Pro Ser Leu Pro Thr
1           5

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(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1912 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: cDNA

- (vi) ORIGINAL SOURCE:
 - (A) ORGANISM: Rhizoctonia laccase

- (ix) FEATURE:

- (A) NAME/KEY: CDS
 - (B) LOCATION: 85..1671

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTAACGCTTG GTGCCGAGCT CGGATCCACT AGTAACGCGC GCCAGTGTGC TGGAAATTGCG	60
GGCCGCGTCG ACACCTCCCT CAAG ATG CTT TCT AGC ATT ACC CTC CTA CCT Met Leu Ser Ser Ile Thr Leu Leu Pro	111
1 5	
TTG CTC GCT GCG GTC TCA ACC CCC GCC TTT GCT GCC GTC CGC AAC TAT Leu Leu Ala Ala Val Ser Thr Pro Ala Phe Ala Ala Val Arg Asn Tyr	159
10 15 20 25	
AAG TTC GAC ATC AAG AAC GTC AAT GTC GCT CCC GAT GGC TTT CAG CGC Lys Phe Asp Ile Lys Asn Val Asn Val Ala Pro Asp Gly Phe Gln Arg	207
30 35 40	
TCT ATC GTC TCC GTC AAC GGT TTA GTT CCT GGC ACG TTG ATC ACG GCC Ser Ile Val Ser Val Asn Gly Leu Val Pro Gly Thr Leu Ile Thr Ala	255
45 50 55	
AAC AAG GGT GAC ACC TTG CGC ATT AAT GTC ACG AAT CAA CTC ACG GAC Asn Lys Gly Asp Thr Leu Arg Ile Asn Val Thr Asn Gln Leu Thr Asp	303
60 65 70	
CCT AGT ATG CGT CGT GCC ACA ACG ATT CAT TGG CAT GGA TTG TTC CAA Pro Ser Met Arg Arg Ala Thr Thr Ile His Trp His Gly Leu Phe Gln	351
75 80 85	

GCT ACT ACC GCC GAC GAG GAT GGC CCC GCA TTC GTC ACG CAA TGC CCT Ala Thr Thr Ala Asp Glu Asp Gly Pro Ala Phe Val Thr Gln Cys Pro 90 95 100 105	399
ATT GCG CAA AAT TTG TCC TAT ACA TAC GAG ATC CCA TTG CGC GGC CAA Ile Ala Gln Asn Leu Ser Tyr Thr Tyr Glu Ile Pro Leu Arg Gly Gln 110 115 120	447
ACA CGA ACC ATG TGG TAT CAC GCC CAT CTT GCG AGT CAA TAT GTC GAT Thr Gly Thr Met Trp Tyr His Ala His Leu Ala Ser Gln Tyr Val Asp 125 130 135	495
GGA TTG CGA GGC CCT TTG GTC ATC TAT GAT CCA AAC GAC CCA CAC AAG Gly Leu Arg Gly Pro Leu Val Ile Tyr Asp Pro Asn Asp Pro His Lys 140 145 150	543
TCG CGC TAC GAC GTG GAT GCG AGC ACA GTA GTC ATG CTT GAG GAC Ser Arg Tyr Asp Val Asp Asp Ala Ser Thr Val Val Met Leu Glu Asp 155 160 165	591
TGG TAC CAT ACT CCG GCA CCC GTT CTA GAA AAG CAA ATG TTC TCG ACT Trp Tyr His Thr Pro Ala Pro Val Leu Glu Lys Gln Met Phe Ser Thr 170 175 180 185	639
AAT AAC ACC GCT CTG CTC TCT CCT GTT CCG GAC TCG GGT CTT ATC AAT Asn Asn Thr Ala Leu Leu Ser Pro Val Pro Asp Ser Gly Leu Ile Asn 190 195 200	687
GGC AAA GGG CGC TAT GTG GGC GGT CCC GCA GTT CCC CGG TCA GTA ATC Gly Lys Gly Arg Tyr Val Gly Gly Pro Ala Val Pro Arg Ser Val Ile 205 210 215	735
AAC GTA AAA CGT GGG AAA CGA TAT CGC TTG CGC GTA ATC AAC GCT TCT Asn Val Lys Arg Gly Lys Arg Tyr Arg Leu Arg Val Ile Asn Ala Ser 220 225 230	783
GCT ATC GGG TCG TTT ACC TTT TCG ATC GAA GGA CAT AGT CTG ACT GTC Ala Ile Gly Ser Phe Thr Phe Ser Ile Glu Gly His Ser Leu Thr Val 235 240 245	831
ATT GAG GCC GAT GGG ATC CTG CAC CAG CCC TTG GCT GTT GAC AGC TTC Ile Glu Ala Asp Gly Ile Leu His Gln Pro Leu Ala Val Asp Ser Phe 250 255 260 265	879
CAG ATT TAC GCT GGA CAA CGC TAC TCT GTC ATC GTT GAA GCC AAC CAA Gln Ile Tyr Ala Gly Gln Arg Tyr Ser Val Ile Val Glu Ala Asn Gln 270 275 280	927
ACC GCC GCC AAC TAC TGG ATT CGT GCA CCA ATG ACC GTT GCA GGA GCC Thr Ala Ala Asn Tyr Trp Ile Arg Ala Pro Met Thr Val Ala Gly Ala 285 290 295	975
GGA ACC AAT GCA AAC TTG GAC CCC ACC AAT GTC TTT GCC GTA TTG CAC Gly Thr Asn Ala Asn Leu Asp Pro Thr Asn Val Phe Ala Val Leu His 300 305 310	1023
TAC GAG GGA GCG CCC AAC GCC GAA CCC ACG ACG GAA CAA GGC AGT GCT Tyr Glu Gly Ala Pro Asn Ala Glu Pro Thr Thr Glu Gln Gly Ser Ala 315 320 325	1071
ATC GGT ACT GCA CTC GTT GAA GAG AAC CTC CAT GCG CTC ATC AAC CCT Ile Gly Thr Ala Leu Val Glu Glu Asn Leu His Ala Leu Ile Asn Pro 330 335 340 345	1119
GGC GCT CCG GGC GGC TCC GCT CCC GCA GAC GTT TCC CTC AAT CTT GCA Gly Ala Pro Gly Gly Ser Ala Pro Ala Asp Val Ser Leu Asn Leu Ala 350 355 360	1167

ATT GGG CGC AGC ACA GTT GAT GGG ATT CTT AGG TTC ACA TTT AAT AAC Ile Gly Arg Ser Thr Val Asp Gly Ile Leu Arg Phe Thr Phe Asn Asn 365 370 375	1215
ATC AAG TAC GAG GCT CCT TCG TTG CCC ACG CTC TTG AAG ATT TTG GCA Ile Lys Tyr Glu Ala Pro Ser Leu Pro Thr Leu Leu Lys Ile Leu Ala 380 385 390	1263
AAC AAT GCG AGC AAT GAC GCC GAT TTC ACG CCA AAT GAG CAC ACT ATC Asn Asn Ala Ser Asn Asp Ala Asp Phe Thr Pro Asn Glu His Thr Ile 395 400 405	1311
GTA TTG CCA CAC AAT AAA GTT ATC GAG CTC AAT ATC ACC GGA GGT GCA Val Leu Pro His Asn Lys Val Ile Glu Leu Asn Ile Thr Gly Gly Ala 410 415 420 425	1359
GAC CAC CCT ATC CAT CTC CAC GGC CAT GTG TTT GAT ATC GTC AAA TCA Asp His Pro Ile His Leu His Gly His Val Phe Asp Ile Val Lys Ser 430 435 440	1407
CTC GGT ACC CCG AAC TAT GTC AAC CCG CCA CGC AGG GAC GTA GTT Leu Gly Gly Thr Pro Asn Tyr Val Asn Pro Pro Arg Arg Asp Val Val 445 450 455	1455
CGT GTC GGA GGC ACC GGT GTG GTA CTC CGA TTC AAG ACC GAT AAC CCA Arg Val Gly Gly Thr Gly Val Val Leu Arg Phe Lys Thr Asp Asn Pro 460 465 470	1503
GCG CCA TGG TTT GTT CAC TGC CAC ATT GAC TGG CAC TTG GAG GCT GGG Gly Pro Trp Phe Val His Cys His Ile Asp Trp His Leu Glu Ala Gly 475 480 485	1551
CTC GCA CTT GTC TTT GCC GAG GCC CCC AGC CAG ATT CGC CAG GGT GTC Leu Ala Leu Val Phe Ala Glu Ala Pro Ser Gln Ile Arg Gln Gly Val 490 495 500 505	1599
CAG TCG GTC CAG CCC AAC AAT GCC TGG AAC CAG CTC TGC CCC AAG TAC Gln Ser Val Gln Pro Asn Asn Ala Trp Asn Gln Leu Cys Pro Lys Tyr 510 515 520	1647
GCG GCT CTT CCT CCC GAT TTG CAG T Ala Ala Leu Pro Pro Asp Leu Gln 525	1672

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 529 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Met Leu Ser Ser Ile Thr Leu Leu Pro Leu Leu Ala Ala Val Ser Thr 1 5 10 15
Pro Ala Phe Ala Ala Val Arg Asn Tyr Lys Phe Asp Ile Lys Asn Val 20 25 30
Asn Val Ala Pro Asp Gly Phe Gln Arg Ser Ile Val Ser Val Asn Gly 35 40 45
Leu Val Pro Gly Thr Leu Ile Thr Ala Asn Lys Gly Asp Thr Leu Arg 50 55 60

Ile Asn Val Thr Asn Gln Leu Thr Asp Pro Ser Met Arg Arg Ala Thr
 65 70 75 80

Thr Ile His Trp His Gly Leu Phe Gln Ala Thr Thr Ala Asp Glu Asp
 85 90 95

Gly Pro Ala Phe Val Thr Gln Cys Pro Ile Ala Gln Asn Leu Ser Tyr
 100 105 110

Thr Tyr Glu Ile Pro Leu Arg Gly Gln Thr Gly Thr Met Trp Tyr His
 115 120 125

Ala His Leu Ala Ser Gln Tyr Val Asp Gly Leu Arg Gly Pro Leu Val
 130 135 140

Ile Tyr Asp Pro Asn Asp Pro His Lys Ser Arg Tyr Asp Val Asp Asp
 145 150 155 160

Ala Ser Thr Val Val Met Leu Glu Asp Trp Tyr His Thr Pro Ala Pro
 165 170 175

Val Leu Glu Lys Gln Met Phe Ser Thr Asn Asn Thr Ala Leu Leu Ser
 180 185 190

Pro Val Pro Asp Ser Gly Leu Ile Asn Gly Lys Gly Arg Tyr Val Gly
 195 200 205

Gly Pro Ala Val Pro Arg Ser Val Ile Asn Val Lys Arg Gly Lys Arg
 210 215 220

Tyr Arg Leu Arg Val Ile Asn Ala Ser Ala Ile Gly Ser Phe Thr Phe
 225 230 235 240

Ser Ile Glu Gly His Ser Leu Thr Val Ile Glu Ala Asp Gly Ile Leu
 245 250 255

His Gln Pro Leu Ala Val Asp Ser Phe Gln Ile Tyr Ala Gly Gln Arg
 260 265 270

Tyr Ser Val Ile Val Glu Ala Asn Gln Thr Ala Ala Asn Tyr Trp Ile
 275 280 285

Arg Ala Pro Met Thr Val Ala Gly Ala Gly Thr Asn Ala Asn Leu Asp
 290 295 300

Pro Thr Asn Val Phe Ala Val Leu His Tyr Glu Gly Ala Pro Asn Ala
 305 310 315 320

Glu Pro Thr Thr Glu Gln Gly Ser Ala Ile Gly Thr Ala Leu Val Glu
 325 330 335

Glu Asn Leu His Ala Leu Ile Asn Pro Gly Ala Pro Gly Ser Ala
 340 345 350

Pro Ala Asp Val Ser Leu Asn Leu Ala Ile Gly Arg Ser Thr Val Asp
 355 360 365

Gly Ile Leu Arg Phe Thr Phe Asn Asn Ile Lys Tyr Glu Ala Pro Ser
 370 375 380

Leu Pro Thr Leu Leu Lys Ile Leu Ala Asn Asn Ala Ser Asn Asp Ala
 385 390 395 400

Asp Phe Thr Pro Asn Glu His Thr Ile Val Leu Pro His Asn Lys Val
 405 410 415

Ile Glu Leu Asn Ile Thr Gly Gly Ala Asp His Pro Ile His Leu His

420 425 430
Gly His Val Phe Asp Ile Val Lys Ser Leu Gly Gly Thr Pro Asn Tyr
435 440 445
Val Asn Pro Pro Arg Arg Asp Val Val Arg Val Gly Gly Thr Gly Val
450 455 460
Val Leu Arg Phe Lys Thr Asp Asn Pro Gly Pro Trp Phe Val His Cys
465 470 475 480
His Ile Asp Trp His Leu Glu Ala Gly Leu Ala Leu Val Phe Ala Glu
485 490 495
Ala Pro Ser Gln Ile Arg Gln Gly Val Gln Ser Val Gln Pro Asn Asn
500 505 510
Ala Trp Asn Gln Leu Cys Pro Lys Tyr Ala Ala Leu Pro Pro Asp Leu
515 520 525
Gln

What we claim is:

1. A nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at 5 pH between about 6.0 and 8.5.
2. The fragment of Claim 1 which comprises a sequence encoding a *Rhizoctonia solani* laccase.
- 10 3. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.
- 15 4. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 4.
- 20 5. The fragment of Claim 1, which comprises a nucleic acid sequence encoding a protein containing one or more of the amino acid sequences depicted in SEQ. ID NOS. 5, 6, 7, 8, 9, 10, 11, or 12.
- 25 6. The fragment of Claim 1 which comprises a nucleic acid sequence encoding the amino acid sequence depicted in SEQ ID NO. 14.
7. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.
- 30 8. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 3.

9. The fragment of Claim 1, which comprises the nucleic acid sequence depicted in SEQ. ID. NO. 13.
10. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21141.
5
11. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21142.
- 10 12. The fragment of Claim 1, which comprises the nucleic acid sequence encoding the laccase produced by RS 22.
13. The fragment of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21156.
15
14. A substantially pure *Rhizoctonia* laccase enzyme which functions optimally at a pH between about 6.0-8.5.
15. The enzyme of Claim 14 which is a *Rhizoctonia solani* laccase.
20
16. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO. 2, or a sequence with at least 80% homology thereto.
25
17. The enzyme of Claim 14 which comprises the sequence depicted in SEQ ID NO 4, or a sequence with at least 80% homology thereto.
- 30 18. The enzyme of Claim 14 which comprises one or more of the peptide sequences depicted in SEQ ID NOS.5, 6, 7,

8, 9, 10, 11 or 12, or a sequence with at least 80% homology to one or more of these peptides.

19. The enzyme of Claim 14 which comprises the sequence
5 depicted in SEQ ID NO 14, or a sequence with at least 80% homology thereto.

20. A recombinant vector comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia*
10 laccase which functions optimally at pH between about 6.0-8.5.

21. The vector of Claim 20 in which the fragment is operably linked to a promoter sequence.

15 22. The vector of Claim 21 in which the promoter is a fungal or yeast promoter.

23. The vector of Claim 22 in which the promoter is the
20 TAKA amylase promoter of *Aspergillus oryzae*.

24. The vector of Claim 22 in which the promoter is the glucoamylase (gluA) promoter of *Aspergillus niger* or *Aspergillus awamsii*.

25 25. The vector of Claim 21 which also comprises a selectable marker.

26. The vector of Claim 25 in which the selectable marker
30 is the amdS marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

27. The vector of Claim 25 in which the selectable marker is the pyrG marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamorii*, or *Aspergillus oryzae*.

5 28. The vector of Claim 21 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the amdS or pyrG marker of *Aspergillus nidulans* or *Aspergillus oryzae*.

10 29. A host cell comprising a heterologous nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase which functions optimally at pH between about 6.0-8.5.

15 30. The host cell of Claim 28 which is a fungal cell.

31. The host cell of Claim 30 which is an *Aspergillus* cell.

20 32. The host cell of Claim 29 in which the fragment is integrated into the host cell genome.

33. The host cell of Claim 29 in which the fragment is contained on a vector.

25 34. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.

35. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO: 4.

36. The host cell of Claim 29 which comprises a fragment containing a sequence encoding the amino acid sequence depicted in SEQ ID NO: 14.
- 5 37. The host cell of Claim 29 which comprises a fragment containing a sequence encoding one or more of the amino acid sequences depicted in SEQ ID NOS.: 5, 6, 7, 8, 9, 10, 11, or 12.
- 10 38. A method for obtaining a laccase enzyme which functions optimally at a pH between about 6.0-8.5 which comprises culturing a host cell comprising a nucleic acid fragment containing a nucleic acid sequence encoding a *Rhizoctonia* laccase enzyme which functions optimally at a pH between 15 about 6.0-8.5, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
- 20 39. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.
- 25 40. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.
- 30 41. A method for oxidizing dyes which comprises contacting the dye with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

42. A method of polymerizing a phenolic compounds which comprises contacting the phenolic compound with a *Rhizoctonia* laccase which functions optimally at a pH between about 6.0-8.5.

5

1 ACCGGTCACACCAAGACATCGGATGAAAACGGAAAGTGTATGCCCATTTGACGTCCTGGGC 60
 61 AACCACTGTTCACTCGCGAGCTAACATGGCGACGTATAAGAACGAGAATGGCC 120
 121 AGATTTCGATATCCCCTCTCGTCCTGGTTTGCTCTCGGCTTGCCTCTAATGGCGGGC 180
 181 CACTTTCCCTTGTCTCGGTTCTCGCTCTTGTTCCTGGCTGTCTGGCGCACCGTCTGAGTA 240
 4 T F L V S L F V S A V L A R T V E Y 24
 241 CGGCCTTGAAGATTAGTGTATGGGGAGATAGGCTCCTGACCGGTGTTAACCGCTTAATGGCGACTTT 300
 1 24 G L K I S D G E I A P D G V K R N A T L 44
 2 / 301 GGgtacggcactcccttgtaatccaaacaattcaaggtttctgatgcttggtcagtaATTGGA 360
 44 V N G 47
 3 61 GGGTATCCGGTCACTCACTTGTCCAAACAAGGGGATACTCTCAAAGTCAAAGGTCCAA 420
 47 G Y P G P L I F A N K G D T L K V K V Q 67
 421 ACAAGCTCACGAAATCCTGAGATGTATCGCACCACTTCCATCgtatgttcgttatc 480
 67 N K L T N P E M Y R T T S I 81
 481 tacttaatacatccgtcgctaaataatctttagCATGGCACGGTCTTACAAACATAGAA 540
 81 H W H G L L Q H R 90

FIG. 1A

541	ACGCCGACGACCGCTCCCTCGTCGTCACTCAGgttaggattctggaaaggttggccctga	600
90	N A D D G P S F V T Q	102
601	actctctgttaaccgacaaccggatgtcaccaggccgttGCCCAGTTGTTCCACGGGAGTCGTAT	660
102	C P I V P R E S Y	111
661	ACTTACACCATAACCTCTGGACGATCAAACCGGAACCTATGGTACCATAGCCACTTGAGT	720
111	T Y T I P L D Q T G T Y W Y H S H L S	131
721	TCGCAATAACGTTGATGGTCTCGAGGGCCCGCTGGTAATCTgtgagtatcttgacttgtct	780
131	S Q Y V D G L R G P L V I	144
781	actgaaggcaacgagactaaaacaaggcgattcacagATGgttcgtctcccttatt	840
144	Y	145
841	tagctctggatcttcatttcacgttaatacatgtatgatAGTCCCAGGATCCTCACAGGCG	900
144	D P K D P H R R	152
901	TTTGTATGATGTTGACGATGAGAAAGACCGTCTGATCATGGTGACTGGTATCATGAATC	960
152	L Y D V D D E K T V L I G D W Y H E S	172
961	GTCCAAGGCAATCCCTTGCTTCGGTAACATTACCCGACAGtaaagtgtatacatgccggtcc	1020
172	S K A I L A S G N I T R Q	185

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1021	cagaaaaattcttaatttcattacaggGCGACCCGGTCTGCCACCATCAACGG 185	R P V S A T I N G	194
1081	CAAAGGTGATTGACCCTGACAAACACTCCTGCCAACCCAGATACTCTGTACACCCCTCAA 194	K G R F D P N T P A N P D T L Y T L K	214
1141	GGTCAAGGGAGGGAAAGGGTACATCGTCTGGTGTCTCATCAATTAGCTCGGAGATCGCTTCGTT 214	V K R G K R Y R L R V I N S S E I A S F	234
1201	CCGGATTCAAGTGTGGAAGGGTACAAGGTGACTGTGATTGCTGCCGATGGCGTCTTACCAA 234	R F S V E G H K V T V I A A D G V S T K	254
1261	ACCGTATCAGGTGATGCCATTGATATTCTAGCAGGACAGGGCATAGATTGGTCTCGTcgtaag 254	P Y Q V D A F D I L A G Q R I D C V	272
1321	tgtcgccgaaccacatctggagctcaagaatgttgtatcatggcgcttataggTGGAGGGC 272	V E A	275
1381	GAACCCAAGAACCGACACATACTGGATCAACGGACCCCTGACCAACGTCGCCAAACAAGAC 275	N Q E P D T Y W I N A P L T N V P N K T	295
1441	CGCTCAGGGCTCTCTCTGTATTAGGGGATCGTGGCCGTACCCCTCCAAAGGGCCC 295	A Q A L L V Y E D R P Y H P P K G P	315
1501	GTATCGCAAGTGGAGGGTCTCTGAGGGATCATCAAGTACTGGAAATCACAAAGCACAAGCA 315	Y R K W S V S E A I I K Y W N H K H K H	335

F-G.1C

1561 CGGACGTGGTTGGACATGGAGCTCGGATGATCGAGGGTAGCCA 1620
 335 G R G L L S G H G L K A R M I E G S H 340

1621 TCATCTGCATTGGCAGGCCGTCGTTAACGCCAGAATGAGACCACACTGTTGTAATGGA 1680
 340 H L H S R S V V K R Q N E T T V V M D 350

1681 CGAGGCAAGCTCGTtgaagtaccatatttaaaagtgggtttcgaaatacttatt 1740
 350 E S K L V

1741 tcaacttttttttagCCAACTGGATAACCCGGCGCTGCATGGGTCTAAACCTGCTGACC 1800
 350 P L E Y P G A A C G S K P A D 365

1801 TCGTCTGGATCTCACTTTGGTTGGtatgttagccaaatcgcccatatacaggataactg 1860
 365 L V L D L T F G L 374

1861 aatatttgtttgtgcgttagAAACTTGGCTACCGGCACGGATGATCAACGGTATCCCAT 1920
 365 N F A T G H W M I N G I P 387

4 / 374 1921 ACGAGTCTCCCCAACATTGCTCAAGATCCTCACTGAGGACGGGGTTACCG 1980
 387 Y E S P K I P T L L K I L T D E D G V T 407

1981 AGTCTGACTTgtatgttcggatcttcgttatcgtgcactcgactcgctgg 2040
 407 E S D F 411

2041 gggaaatttagCACCAAGGAGGACACAGTCATACTCCCGAAGAACAAATGCATCGAAT 2100
 411 T K E E H T V I L P K N K C I E 427

FIG. 1D

2101 TCAACATCAAGGGAAACTCGGGTATTCCCATTAAGCACCCCGTACATCTTCACGGTgtaa 2160
 427 F N I K G N S G I P I T H P V H L H G 446
 2161 gttgcataatcggtatggtttacgataactaaaggctcatcaacttttagCACACTGGGATGT 2220
 446 H T W D V 451
 2221 CGTACAATTGGCAACACCCAAATTATGTCATAATCCTCCCCGTAGGGACGTTGGTGG 2280
 451 V Q F G N N P P N Y V N P P R R D V V G 471
 2281 CTCTACAGATGGGGTGTGAGGATTCAAGGACCAATCCAGGACCGTGGTTCCT 2340
 471 S T D A G V R I Q F K T D N P G P W F L 491
 2341 GCAC TGgtgcgtcggccatcggtccgttatggttatgtttcttaatacgtcccattttat 2400
 491 H C 493
 2 / 2401 tagCCATATTGACTGGCATCTTGAGGAGGGTTTCGCAAGtgtgagttactgagaccctaagtgc 2460
 493 H I D W H L E E G F A 504
 2461 tactcggtcttactgattaccgcattatgcgtatgcgtcttagtGGTGTGCTGAAGGCCCG 2520
 504 M V F A E A P 511
 2521 AAGCCGTCAGGGAGGTCCAAGAGGGTGGCGTGGGACTCTCAGTGGAAAGGGCTGTGTG 2580
 511 E A V K G G P K S V A V D S Q W E G L C 531
 2581 GCAAGTACGACAACTGGCTAAATCAAATCCGGGCCAGGTGTAGGGTATCGCAGGCCACA 2640
 531 G K Y D N W L K S N P G Q L * 545

FIG. 1E

2641 TGGGTGATTGAAAGTGTGCATCTTGTCCCTATAACCGGCTCTATAACGGGTGTC 2700

2701 CCAGTAAGTGTAGCCCCAATTTCAGCCGAGACAGATATTAGTGGACTCTACTTG 2760

2761 GTCCCCATTTGACGCACATCGTTGCATCAAACCTGCTTTTATCGTCCCCTCTTTGTAATTG 2820

2821 TGTTGCTGTAATGTATCG 2828

F I G. 1F

1 AAGCTTCCGGCATGGATTGCCATTGTATTGT 180

181 AAACAAGTTACGAGAAAACAATAGATCAGTTTGCCGAATCGGATGGCTTGAAACCGGA 240

241 AGTACCGGATGGCCGATCCGAGTCGAATGAAATTAAACGCATCTGAAACGGGACCCCTGAGTCG 300

301 AGGCACCCGGCCGGCCTGGCCGTATAAAGTCACTTGTGCCAAACTAGCACTTTTCAATTCC 360

361 CCCTTTCTTCTCCTCGTCTTCTCTCTATGGCTCGGTGACTACTCACTCTTG 420
1 M A R S T T S L F 10

421 CACTGTCTCTGGCCGACCGGCCCTGGCTCGAGTCGTTGACTATGGGTTGATGGCTA 480
10 A L S L A A P A L V D Y G F D V A 30

481 ATGGGGCAGTTGCTCCGGATGGTGTAAACAAAGGAACGGAACGGGTTCTCGgttagtttagctgtaa 540
30 N G A V A P D G V T R N A V L 45

541 gatggtgttatatgctggttgcctaacggaaatgtcagTCATGGTCAATGGCTCGCTTCCCTGGTCCA 600
45 V N G R F P G P 53

601 TTGATCACCGCCAAACAAGGGGGATAACACTTAAATCACCTTACACTAAACTCTCCGAT 660
53 L I T A N K G D T L K I T V R N K L S D 73

FIG. 2A

661 CCAAATGCGAAGGAGCACGACCATCGtttagtactccctcatctgtttgaaacttt 720
 73 P T M R S T T I 82

721 ctcatcttttgaaagCACTGGCACGGTCTGCTCCAAACACAGGACGGCAGAACAGATGG 780
 82 H W H G L L Q H R T A E E D G 97

781 CCCGGCCTTTGTAACCCAGgtatgccttatcctatcgctgtcccccggcgctctcc 840
 97 P A F V T Q 103

841 ctgactcggccattcttagtGCCGATTCCCGATTCCGAAAGAATCGTACACCTATACTGCC 900
 103 C P I P P Q E S Y T Y T M P 117

901 GCTCGGGAACAGACCGGCACGTATTGGTACCTGAGCTCCCACTATGTGGA 960
 117 L G E Q T G T Y W Y H S H L S S Q Y V D 137

961 CGGGTTGGTGGCCATCGTTATTGtaagtcttcattaacccttattttggctatgg 1020
 137 G L R G P I V I 145

1021 ctgattgtgacgtcggttagATGgtcggtggctccacaagaaggtagcagcccttga 1080
 145 Y 145

1081 agctaactttttccagACCCCCACGACCCGTACAGAAACTACTATGATGTCGACCGACGA 1140
 145 D P H D P Y R N Y D V D D E 160

1141 CGGTACGGTCTTTACTTTAGCAGACTGGTACCAACGCCGTCGGAGGCTATCATGCCAC 1200
 160 R T V F T L A D W Y H T P S E A I I A T 180

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1201 CCACGATGTCTTGAAACGtacggtaatcccttagttttccctgggtcactt 1260
 180 H D V L K T 185

1261 ctatcaggATCCCCGACTCGGGTACATCAACGGCAAAGGCAAATAcgATCCCTGCTTCGG 1320
 185 I P D S G T I N G K Y D P A S 202

1321 CTAACACCAACACGACACTCGAGAACCTACTACACTCTCAAGTCAAAGTCAAACGGCAAGC 1380
 202 A N T N N T L E N L Y T L K V R G K 222

1381 GGTATCGCCTGAGGATTATCAACGCCTGGCCATCGCTTCGCTTCGGTTGGCGCAGG 1440
 222 R Y R L R I I N A S A I A S F R F G V Q 242

1441 GCCACAAAGTGCACGATCATCGAGGCTGATGGGCTGATGGGTATAAGCTGAGGTCGATG 1500
 242 G H K C T I I E A D G V L T K P I E V D 262

9 / 1501 CGTTTGATATTCTAGCAGGCCAGGGTATAAGCTGCATCGtaagtctaccatatgccttgt 1560
 262 A F D I L A G Q R Y S C I 275

1561 gtggagataagaacctgactgaatgtatgcgtccaaatagTGAAGGCCAACGATCC 1620
 275 L K A D Q D P 282

1621 TGATTCTACTGGATAAAATGCGCCAATCACAAACGTTCTCAACACGTTCCAGGCAT 1680
 282 D S Y W I N A P I T N V L N T N V Q A L 302

1681 GCTAGTGTATGAAAGATGACAAGCGGTCTACTACTACCCCTGGAAAGCCGGTTTGACATG 1740
 302 L V Y E D D K R P T H Y P W K P F L T W 322

1741 GAAGATATCAAATGAAATTCACTTCAGTACTGGCAGGCCAACAGCACGGTCCGCACGGTCACAA 1800
 322 K I S N E T I Q Y W Q H K H G S H G H K 342

1801 GCGAAAGGGCATCATCATAAAGTCCGGCCATGGAGGTGTTGAGCTCCAG 1860
 342 G K G H H K V R A I G G V S G L S S R 362

1861 GGTAAAGAGCCGGGAGTGACCTATCGAAGAAGGCTGTGAGTTGGCTGCTGCACTCGT 1920
 349 V K S R A S D L S K K A V E L A A L V 349

1921 TCGGGGTGAGGCCGGAGTGGACAAGAGGGCAGAAATGAGGATAATTGACTATTGTATTGGA 1980
 349 A G E A E L D K R Q N E D N S T I V L D 361

1981 TGAGACCAAGCTTATTgttaagtcccttaatttttcggtgtcacggaaggctaaccgcg 2040
 361 E T K L I 361

10/21 2041 taatagCCGTTCAACCTGGTGCACCGGGGGCTCCAGACCAGCTGACGTCGGTC 2100
 361 P L V Q P G A P G S R P A D V V V 379

2101 CCTCTGGACTTGGCTCgtatggcttcttgttattcgtccggaaatgcaactgattt 2160
 379 P L D F G L 385

2161 gggtggctatagaACTTGGCAACGGACTGTGACGATAAACAAATGTCCTACTCCCC 2220
 385 N F A N G L W T I N N V S Y S P 401

2221 TCCGGATGTCCCTTACTCTCAAGATCTTGACCGACAAAGACAAAGTCGACGGCTTCCTGA 2280
 401 P D V P T L L K I L T D K V D A S D 421

FIG. 2D

2281 CTTgtaggttcccttttcaaaacttagctactgacatcaaagtgaacgtcagCACC 2340
 421 F T 423

2341 GCCGATGAAACACAGTATTCTCCAAAGAACCAAGTGTGAGTTGCACATCAAAGGGA 2400
 423 A D E H T Y I L P K N Q V V E L H I K G 453

2401 CAGGCTTGGAAATCGTACACCCCTTCATCTGCATGGCgtacgttttcacactgtt 2460
 453 Q A L G I V H P L H L H G 466

2461 ccagctccatttctctaacaacactcctgcgataaggCATGCCGTTGCACGTCGTCCAAATTGG 2520
 466 H A F D V V Q F G 475

2521 CGACAACGCTCCAAACTACGTGAACCCCTCCGGTAGGGATGTAGCTAGGCCGTAACTGATGC 2580
 475 D N A P N Y V N P P R R D V V G V T D A 495

2581 TGGAGTCCGTATCCAGTTAGAACCGATAACCCGGCCCTGGTTCCCTCATTGGtatgc 2640
 495 G V R I Q F R T D N P G P W F L H C 513

2641 tcttcattcccccaccgcgttgtttactttatgttttaccccttgcgattttagCCACATTGA 2700
 513 H I D 516

2701 TTGGCACTTGGAAAGGATTGGCTAGtaagtattttatccggatccgggg 2760
 516 W H L E E G F A 524

2761 gatgctaaccaaagggttgttttaagtGGTATTGCGCCGAAAGATATCAAGAA 2820
 524 M V F A E A P E D I K K 536

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2821 AGGCTCTCAGAGTGTCAAGCCTGACGGACAATGGAAGAACTATGCCAGAAGTATGAGAA 2880
536 G S Q S V K P D G Q W K K L C E K Y E K 556

2881 GTTGCCCTGAAGGCACTGCAGTGAAGTGTGCAGTTCCCATTCGGAAACTGGCTCACTAT 2940
556 L P E A L Q * 562

2941 TCCTTTGCATAATTGGACTTTGGACATTGGACTATGCCATTGGTC 3000

3001 ACACCGGGAACTAAGCCGAATTC

F I G. 2F

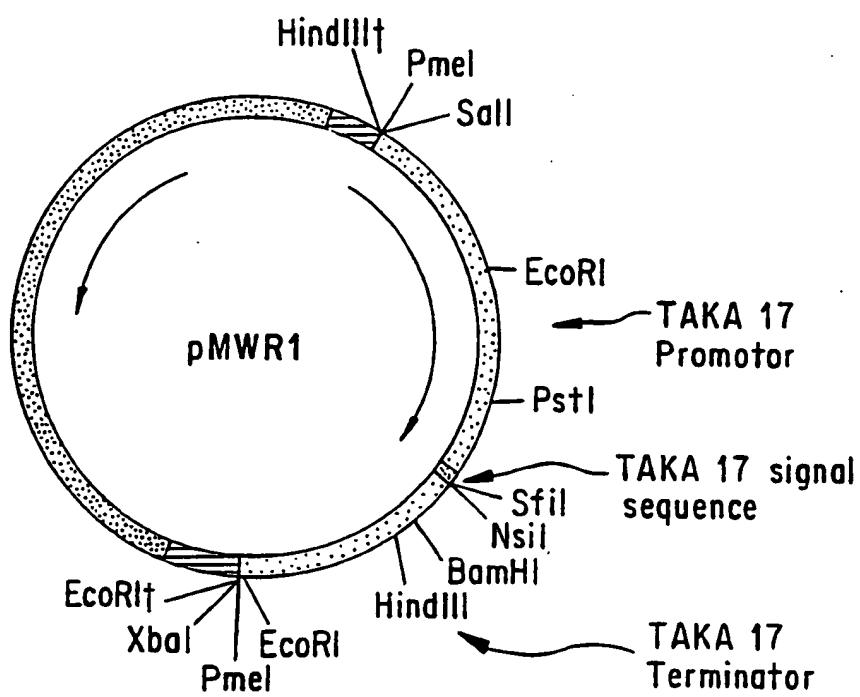


FIG. 3

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5'	ATG	TCT	TCT	AGC	ATT	ACC	CTC	CTA	CCT	TTC	CTC	GCT	GCG	GTC	TCA	ACC	CCC	GGC	132
M	L	S	S	I	T	L	L	P	L	A	A	V	S	T	P	A			
	141		150		159		168		177										186
F	A	A	V	R	N	Y	K	F	D	I	K	N	V	N	V	A	P		
	195		204		213		222		231										240
D	G	F	Q	R	S	I	V	S	V	N	G	L	V	P	G	T	L		
	249		258		267		276		285										294
I	T	A	N	K	G	D	T	L	R	I	N	V	T	N	Q	L	T		
	303		312		321		330		339										348
D	P	S	M	R	R	A	T	T	I	H	W	H	G	L	F	Q	A		

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SUBSTITUTE SHEET (RULE 26)

FIG. 4A

FIG. 4B

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SUBSTITUTE SHEET (RULE 26)

FIG. 4C

CAA	CGC	TAC	TCT	GTC	ATC	GTT	GAA	GCC	AAC	CAA	ACC	924	933	942			
Q	R	Y	S	V	I	V	E	A	N	Q	T	A	N	Y	W	I	
951	960	969	978	987	996												
CGT	GCA	CCA	ATG	ACC	GTT	GCA	GGA	GCC	GGG	ACC	AAT	GCA	AAC	TTG	GAC	CCC	ACC
R	A	P	M	T	V	A	G	A	G	T	N	A	N	L	D	P	T
1005	1014	1023	1032	1041	1050												
AT	GTC	TTT	GCC	GTA	TTC	CAC	TAC	GAG	GGA	GCG	CCC	AAC	GCC	GAA	CCC	ACG	ACG
N	V	F	A	V	L	H	Y	E	G	A	P	N	A	E	P	T	T
1059	1068	1077	1086	1095	1104												
GAA	CAA	GGC	AGT	GCT	ATC	GGT	ACT	GCA	CTC	GTT	GAA	GAG	AAC	CTC	CAT	GGG	CTC
E	Q	G	S	A	I	G	T	A	L	V	E	E	N	L	H	A	L
1113	1122	1131	1140	1149	1158												
ATC	AAC	CCT	GGC	GCT	CCG	GGC	GGC	TCC	GCT	CCC	GCA	GAC	GTT	TCC	CTC	AAT	CTT
I	N	P	G	A	P	G	G	S	A	P	A	D	V	S	L	N	L

	1167	1176	1185	1194	1203	1212
GCA	ATT	GGG	CGC	AGC	ATA	GAT
						GGG
A	I	G	R	S	T	V
						D
						G
						I
						L
						R
						F
						T
						F
						N
						N
						I
	1221	1230	1239	1248	1257	1266
AAG	TAC	GAG	GCT	CCT	TCG	CCC
						ACG
K	Y	E	A	P	S	L
						P
						T
						L
						K
						I
						L
						A
						N
						N
						A
	1275	1284	1293	1302	1311	1320
AGC	AAT	GAC	GGC	GAT	TTC	ACG
						CCG
S	N	D	A	D	F	T
						P
						N
						E
						H
						T
						I
						V
						L
						P
						H
						N
	1329	1338	1347	1356	1365	1374
AAA	GTT	ATC	GAG	CTC	AAT	ATC
						ACC
K	V	I	E	L	N	I
						T
						G
						G
						A
						D
						H
						P
						I
						H
						L
						H
	1383	1392	1401	1410	1419	1428
GGC	CAT	GTG	TTT	GAT	ATC	GTC
						AAA
G	H	V	F	D	I	V
						K
						S
						L
						G
						T
						P
						N
						Y
						V
						N

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FIG. 4E

		1437	1446	1455	1464	1473	1482										
CCG	CCA	CGC	AGG	GAC	GTA	GTT	CGT	GTC	GGA	GGC	ACC	GGT	GTG	GTA	CTC	CGA	TTC
P	P	R	R	D	V	V	R	V	G	G	T	G	V	V	L	R	F
K	T	D	N	P	G	P	W	F	V	H	C	H	I	D	W	H	L
E	A	G	L	A	L	V	F	A	E	A	P	S	Q	I	R	Q	G
V	Q	S	V	Q	P	N	N	A	W	N	Q	L	C	P	K	Y	A
19 / 21	1599	1608	1617	1626	1635	1644											
GCT	CAG	TCG	GTC	CAG	CCC	AAC	AAT	GCC	TGG	AAC	CAG	CTC	TGC	CCC	AAG	TAC	GCG
A	L	P	P	D	D	L	L	Q	-	-	-	-	-	-	-	-	-

FIG. 4F

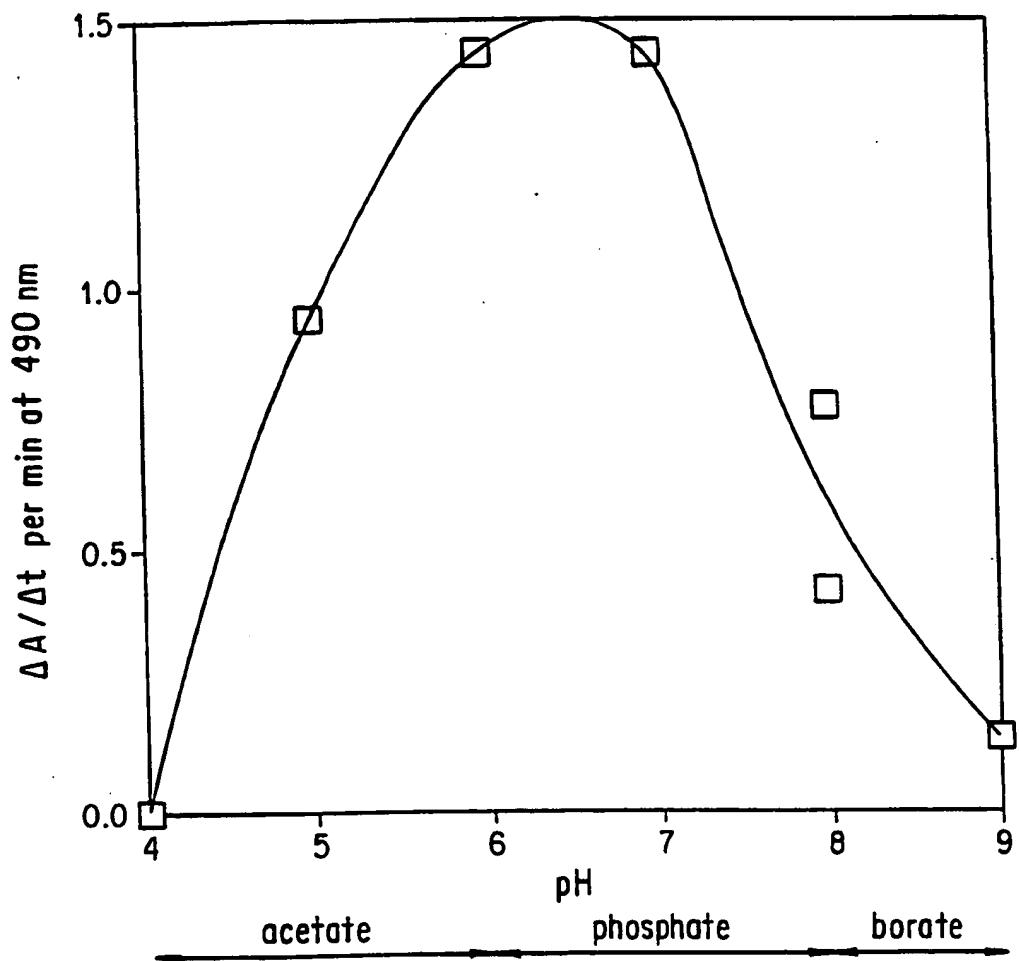


FIG. 5

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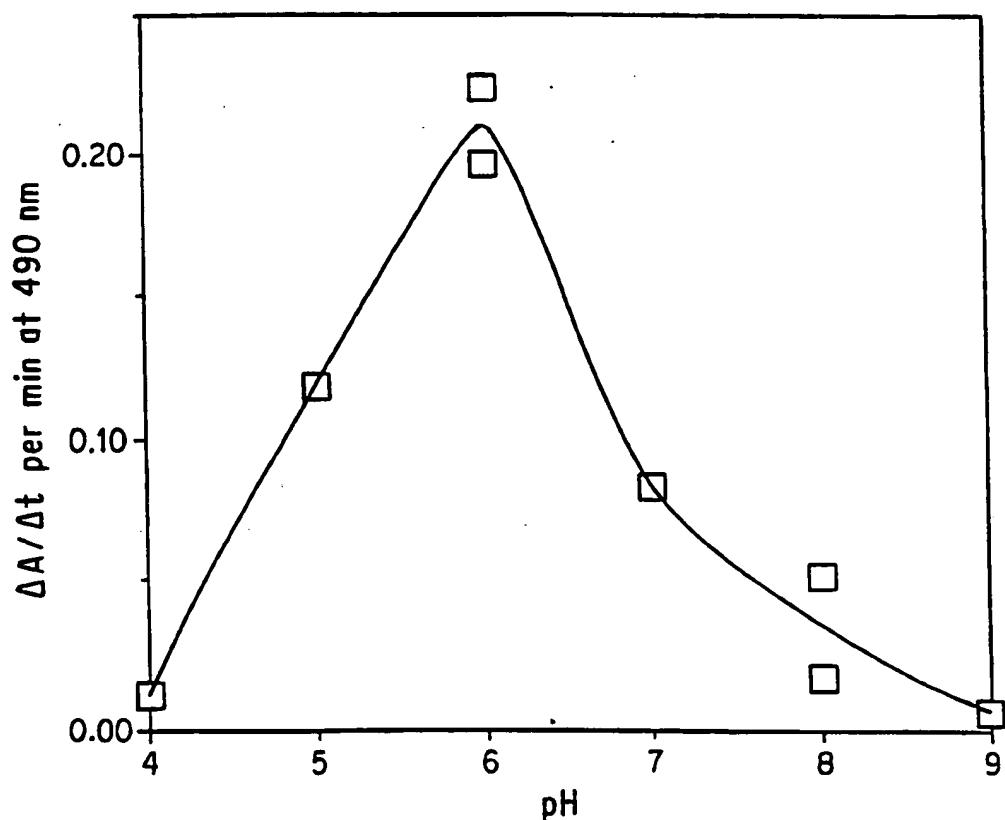


FIG. 6

INTERNATIONAL SEARCH REPORT

Int. Appl. No.
PCT/US 94/10264

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/53 C12N9/02 C12N15/80 D21C5/00 A61K7/06
 C12P7/22 C12N1/19 C09B69/10 // (C12N1/19, C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N D21C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 90, no. 19, 7 May 1979, Columbus, Ohio, US; abstract no. 147536w, BOLLAG J.M. ET AL. 'Characterization of an enzyme from Rhizoctonia praticola which polymerizes phenolic compounds.' page 213 ; see abstract & CAN. JOURNAL MICROBIOL., vol.25, no.2, 1979 pages 229 - 223 ----	14, 43
Y	----- -----	1, 20-24, 39-41

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

24 January 1995

Date of mailing of the international search report

23. 02. 95

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Delanghe, L

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 94/10264

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO,A,92 16633 (NOVO NORDISK) 1 October 1992 see page 3; claims ---	21-24
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Y	EP,A,0 408 803 (ENSO-GUTZEIT OY) 23 January 1991 see claims ---	41
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